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CONTROL OF EPITHELIAL Cl^- SECRETION BY BASOLATERAL OSMOLALITY IN THE EURYHALINE TELEOST *FUNDULUS HETEROCLITUS*

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Summary

Euryhaline teleost fish adapt rapidly to salinity change and reduce their rate of ion secretion on entry to fresh water. Killifish (*Fundulus heteroclitus*) transferred from full-strength sea water to fresh water showed large reductions in plasma $[Na^+]$ and osmolality at 6 h which were corrected by 24 h. To mimic this *in vitro*, a hypotonic shock of 20–70 mosmol kg^{-1} was applied on the basolateral side of opercular epithelia. This hypotonic shock reversibly reduced the short-circuit current (I_{sc}, equivalent to the rate of secretion of Cl^-) in a dose-dependent fashion, with a 40 mosmol kg^{-1} hypotonic shock reducing I_{sc} by 58±4.6 % in 40 min. Similar reductions in [NaCl], but with added mannitol to maintain osmolality, were without effect, indicating that the effect was purely osmotic. Hypotonic inhibition of I_{sc} was accompanied by reductions in epithelial conductance (G_t) but no significant change in transepithelial potential (V_t). The hypotonic inhibition was apparently not Ca^{2+}-mediated because Ca^{2+}-depleted salines, thapsigargin and ionomycin all failed to block the reduction in I_{sc} produced by hypotonic shock. The inhibition was not mediated *via* a reduction in intracellular cyclic AMP level because cyclic AMP levels, measured by radioimmunoassay, were unchanged by hypotonic shock and by 1.0 μmol l^{-1} clonidine (which inhibits I_{sc} by changing intracellular $[Ca^{2+}]$) but were increased markedly by 1.0 μmol l^{-1} isoproterenol, a positive control. The protein tyrosine kinase inhibitor genistein (100 μmol l^{-1}), but not its inactive analogue daidzein, inhibited I_{sc} in normal osmolality but produced a stimulation of I_{sc} after hypotonic shock (and after clonidine treatment). The inhibitory effects of genistein and hypotonicity were not additive, suggesting that the same portion of the I_{sc} was inhibited by both treatments. These data are consistent with a model for Cl^- transport regulation involving tyrosine phosphorylation in cell-swelling-induced inhibition of Cl^- secretion when euryhaline teleosts adapt to fresh water.

Key words: epithelial ion transport, chloride-secreting cell, osmoregulation, cell volume regulation, thapsigargin, cyclic AMP, daidzein, genistein, teleost, killifish, *Fundulus heteroclitus*.

Introduction

Euryhaline teleosts that enter fresh water respond by rapidly reducing the rate of ion secretion and the rate of passive ion loss across the gill and skin epithelia, but the mechanisms by which these events occur are not well understood. It is well known that blood ion levels and osmolality rise when euryhaline teleosts are transferred to higher salinities and that they fall in animals transferred to more dilute solutions. Early experiments involving salt depletion and salt loading demonstrated appropriate increases in Na^+ efflux with salt loading and decreases with salt depletion (for a review, see Maetz, 1974), but these effects were not connected to blood osmolality *per se*. Salinity transfer experiments that examined the acclimation of killifish to sea water demonstrated an elevation of serum osmolality in the first 2 days, after which plasma ion levels and osmolality were corrected (Jacob and Taylor, 1983; Zadunaisky et al., 1995; Marshall et al., 1999).

An increase in [NaCl] and/or hypertonicity on the basolateral side of opercular epithelia *in vitro* (to mimic early events during the adaptation to hypersaline conditions) evokes an immediate increase in the rate of secretion of Cl^- (Zadunaisky et al., 1995). This effect cannot be mimicked by a similar change in the apical bathing solution, suggesting that the cells are responding specifically to a blood parameter. However, because the osmolality of the control Ringer used in the previous experiments was 250 mosmol kg^{-1} and the normal osmolality of the plasma of seawater mummichog is 290–308 mosmol kg^{-1} (Zadunaisky et al., 1995; Marshall et al., 1999), the 'hypertonic' state may actually have been a recovery from an induced hypotonic state. This makes the results difficult to interpret physiologically. Nevertheless, changes in plasma osmolality may be an important early cue that directly affects ion-transporting cells and can affect Cl^- secretion. In turn, augmentation of Cl^- secretion may depend in part on the degree of phosphorylation of the basolateral $Na^+/K^+/2Cl^-$ cotransporter (Flemmer and Forbush, 1999) and/or the apically located cystic fibrosis transmembrane conductance regulator (CFTR) anion channel (Singer et al., 1998). These

findings are consistent with observations of the effects of hypertonicity in mammalian renal medullary cells (for a review, see Burg et al., 1997) that connect hypertonic shock with alterations in cell function and gene expression.

However, euryhaline teleosts also adapt rapidly to hypotonic solutions. In sea water, there is a well-established NaCl secretion mechanism involving a basolateral $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase in series with anion channels that are similar to the CFTR anion channel in the apical membrane, and Cl^- secretion follows this transcellular pathway while Na^+ is secreted passively *via* a paracellular route (for reviews, see Wood and Marshall, 1994; Marshall and Bryson, 1998). Transfer of mummichogs to fresh water produces a sharp decrease in the rate of Na^+ efflux that starts in the first 30 min and declines more slowly over 4–8 h (Potts and Evans, 1967; Motais et al., 1966). The 'instantaneous' reduction that occurs in the first 30 min is followed by a more protracted reduction in the rate of ion loss and augmentation of ion uptake during full adaptation to fresh water (Wood and Marshall, 1994). We have shown that a portion of the rapid reduction in the rate of ion secretion may be mediated by α_2-adrenergic receptors that are activated by the sympathetic nervous system (Marshall et al., 1998c) and that these α_2-receptors act *via* phospholipase C and inositol trisphosphate and intracellular Ca^{2+} (Marshall et al., 1993, 1998c). Neural stimulation *in vitro* did not completely inhibit Cl^- secretion (Marshall et al., 1998c), so other inhibitory mechanisms presumably also contribute.

To examine the possible role of hypotonic shock in the shutdown of Cl^- secretion when teleosts enter fresh water, whole animals were transferred to fresh water and their blood ion levels monitored (to determine the extent of hypotonicity). Opercular epithelia isolated *in vitro* were exposed to basolateral hypotonic shock while electrophysiological aspects of ion transport were measured. Because cell swelling and the phosphorylation state of ion transporters are probably involved, the protein tyrosine kinase inhibitor genistein was applied to initiate a mechanistic investigation of the reduction in the rate of ion secretion in response to hypotonic shock. Preliminary results have been presented elsewhere (Marshall and Bryson, 1997; Marshall et al., 1998a).

Materials and methods

Animals

Adult mummichogs (common killifish, *Fundulus heteroclitus*) of both genders were captured in Antigonish estuary, transferred to indoor holding facilities and adapted to full-strength sea water (salinity 3 g l^{-1}) for at least 10 days at 20–25 °C and ambient photoperiod under artificial light. Fish were fed marine fish food blend (Nutrafin flakes and tubifex worms, R. C. Hagen, Montreal) at a rate of 1.0 g 100 g^{-1} body mass day^{-1}, supplemented twice weekly with frozen brine shrimp.

Salinity transfer

Transfer to fresh water involved the transfer of seawater-acclimated mummichog to dechlorinated, ultraviolet-sterilized Antigonish tapwater ([NaCl], 0.15–0.30 mmol l^{-1}; [Ca^{2+}], 0.04–0.08 mmol l^{-1}; pH 5.5–6.5) in glass aquaria. Pairs of seawater animals were placed into 10 l aquaria of full-strength sea water at 20 °C for 7 days (to acclimate to the new surroundings), and fresh water was then introduced as a flow of water at the same temperature over a period of 10–12 min to complete the salinity transfer. Animals were removed after 6, 12, 18 and 24 h and anaesthetized in buffered (pH 7.1) 1:5000 tricaine methane sulphonate (MS222) in 150 mmol l^{-1} NaCl. Blood was collected from the severed caudal peduncle into heparinized haematocrit tubes. Plasma [Na^+] was measured on triplicate dilutions using atomic absorption spectrophotometry (Varian AA-375).

Epithelial preparation

Animals were anaesthetized in 0.2 g l^{-1} MS222 buffered to pH 7 and killed by decapitation. The paired branchial epithelia were dissected from the medial surface of the left and right opercular bones using fine forceps while the epithelium was kept moist with Cortland's saline (composition given below). The membranes were mounted over a round aperture 0.125 cm^2 in diameter held in place by five insect pins and supported by an open-weave nylon mesh. The 2 mm rim adjacent to the aperture was bevelled and coated with a thin layer of high-vacuum grease to minimize edge damage to the preparation. The insert was mounted in a modified Ussing chamber surrounded by a water jacket controlled at 22±1 °C, stirred vigorously by magnetic fleas and aerated with 99 % O_2/1 % CO_2. Hemichamber volume was 4.0 ml. Voltage asymmetries were zeroed, and solution resistance (approximately 100 Ω) was compensated in the voltage clamp. Transepithelial potential (V_t in mV), transmembrane resistance (R_t, in Ω cm^2, calculated from voltage responses to transepithelial current pulses of 5.0 μA) and short-circuit current (I_{sc}, μA cm^{-2}) were measured using current/voltage clamps (D. Lee Co., Sunnyvale, CA, USA, or WP Instruments DVC 1000). I_{sc} was expressed as positive for secretion of anions, and epithelia were clamped to 0 mV except for short periods to record V_t. Seawater opercular epithelia were deemed acceptable if they developed a steady-state I_{sc} greater than 50 μA cm^{-2} and had an R_t of at least 80 Ω cm^2 to eliminate damaged membranes. Typical values were a steady-state I_{sc} of 125 μA cm^{-2} and an R_t of 100 Ω cm^2.

Experimental design

In each case, paired membranes from one animal were mounted, with one serving as a parallel running control, and allowed to come to a steady-state I_{sc} over approximately 30–40 min. The treatments were then applied, and the membrane was allowed to come to a new steady-state I_{sc}, usually within 30–40 min. The control membrane received the drug vehicle or, in the case of genistein, equimolar treatment with daidzein. In some cases, subsequent treatments were applied and the membranes were again allowed to come to steady state.

Bathing solutions

A modified Cortland's saline (305 mosmol kg^{-1}, pH 7.8) was used to bathe both membrane surfaces symmetrically; its composition was (in mmol l^{-1}): NaCl, 160; KCl, 2.55; $CaCl_2$, 1.56; $MgSO_4$, 0.93; $NaHCO_3$, 17.85; NaH_2PO_4, 2.97; glucose, 5.55. The saline had a pH of 7.8 when equilibrated with a 99 % O_2/1 % CO_2 gas mixture. Hypotonic shock was applied to the basolateral bathing solution by flow-through of 7–10 times the chamber volume using a dilution of the modified Cortland's saline. The standard hypotonic shock applied was a step from 305 to 234 mosmol kg^{-1} (a change of 71 mosmol kg^{-1}), accomplished by flow-through of diluted Cortland's solution (three parts normal Cortland and one part deionized water). V_t measurements made under these asymmetrical conditions (dilute Cortland on the basal side) were not corrected for junction potentials because these were less than 1 mV, as measured against a flowing 3 mol l^{-1} KCl half-cell. The change in solution resistance due to the dilution was also negligible (approximately 2 Ω cm^2). The hypotonic treatment is approximately the same reduction in plasma ion content and osmolality as that 6 h after direct transfer to fresh water.

To test for effects of ion depletion *versus* hypotonicity, an ion-depleted isotonic solution was prepared by the addition of mannitol (71 mmol l^{-1}) to the diluted Cortland to bring the osmolality back to 305 mosmol kg^{-1}. The isotonic ion-depleted Cortland and hypotonic Cortland were applied to paired membranes using the same flow-through protocol, with the latter serving as the positive control.

The low-[Ca^{2+}] regime involved a nominally Ca^{2+}-free basolateral solution and an apical solution with enough Ca^{2+} to maintain the integrity of intercellular junctions in the epithelium (as judged by the maintenance of transepithelial resistance). The basolateral solution was Cortland's saline with 10 μmol l^{-1} $CaCl_2$ and 70 μmol l^{-1} EDTA added instead of the normal Ca^{2+} content; this produced a calculated Ca^{2+} activity of less than 1.0 μmol l^{-1}. The apical solution was Cortland's saline with a reduced Ca^{2+} content, 0.1 mmol l^{-1} Ca^{2+} added instead of the normal 1.56 mmol l^{-1}. These solutions were introduced by flow-through of 7–10 times the chamber volume.

Cyclic AMP radioimmunoassay

Samples of opercular epithelia were dissected, cut into four (approximately 2 mg wet mass) and incubated in 400 μl of Cortland's saline (300 μl for hypotonic treatment) to equilibrate for 30 min. Control membranes were not disturbed, while test membranes were treated with 1.0 μmol l^{-1} clonidine, 1.0 μmol l^{-1} isoproterenol, 10 μmol l^{-1} forskolin, hypotonic shock (addition of deionized water to 400 μl) or 10 μmol l^{-1} forskolin plus hypotonic shock. After incubation for 10 min at 20 °C, tissue samples were placed in boiling water for 5 min and processed according to the low-range acetylation protocol for cyclic AMP radioimmunoassay, but with all volumes halved (Biotrak RPA509, Amersham International). The tissue pellets were assayed for protein content using a modified Lowry technique (Lowry micromethod kit 690-A, Sigma Scientific). The cyclic AMP content was expressed as pmol mg^{-1} protein.

Pharmaceuticals

The phytoestrogen genistein, 5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-penzopyran-4-one, which inhibits protein tyrosine kinases, and its inactive analogue daidzein, 7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-penzopyran-4-one (both from Sigma), were dissolved in dimethylsulphoxide (DMSO) and added at a final concentration of 100 μmol l^{-1}. The final DMSO content was less than 0.2 μl ml^{-1} and had no effect on electrophysiological variables. Isoproterenol and forskolin (Sigma) were dissolved in saline.

Statistical analyses

Data are presented as the mean ±1 S.E.M. Comparisons between treatments were performed using paired or unpaired *t*-tests (as appropriate) with an α value of 0.05 to indicate the minimum statistical significance level.

Results

Mummichogs transferred from full-strength sea water to Antigonish tapwater showed a significant reduction in plasma [Na^+] at 6 h (Fig. 1), but this had recovered by 12 h and then remained stable until 24 h after transfer. The reduction was approximately 29 % or 59 mmol l^{-1} Na^+. Assuming equal reductions in anion complement and taking the activity coefficient to be 150–200 mmol l^{-1}, the reduction represents a decrease of approximately 94 mosmol kg^{-1}. Clearly, there is sufficient reduction in plasma ionic constituents to produce a hypotonic shock to the cells.

Opercular epithelia mounted *in vitro* in membrane chambers were selectively exposed to hypotonic solutions with stepwise decreases in osmolality by flow-through of diluted samples of the serosal bathing solution. The mucosal bathing solution was unchanged. A reduction in serosal bathing solution osmolality

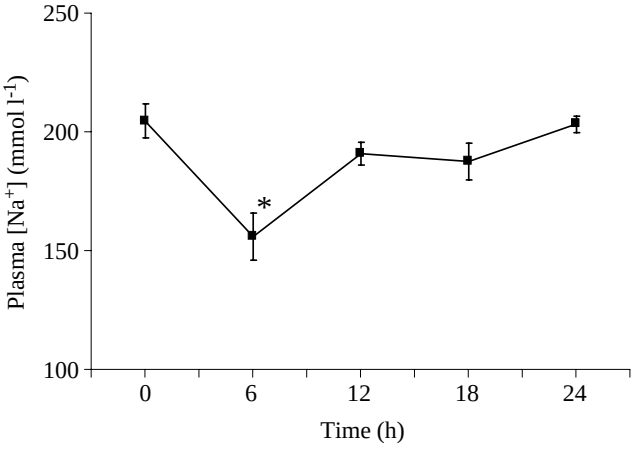

Fig. 1. Plasma [Na^+] of mummichogs adapted to full-strength sea water before transfer to fresh water (at time 0) and at 6, 12, 18 and 24 h after transfer. Plasma [Na^+] was significantly reduced at 6 h, compared with initial levels (*P<0.001, unpaired *t*-test, N=6), but had recovered to control levels by 12 h after transfer. Values are means ± S.E.M.

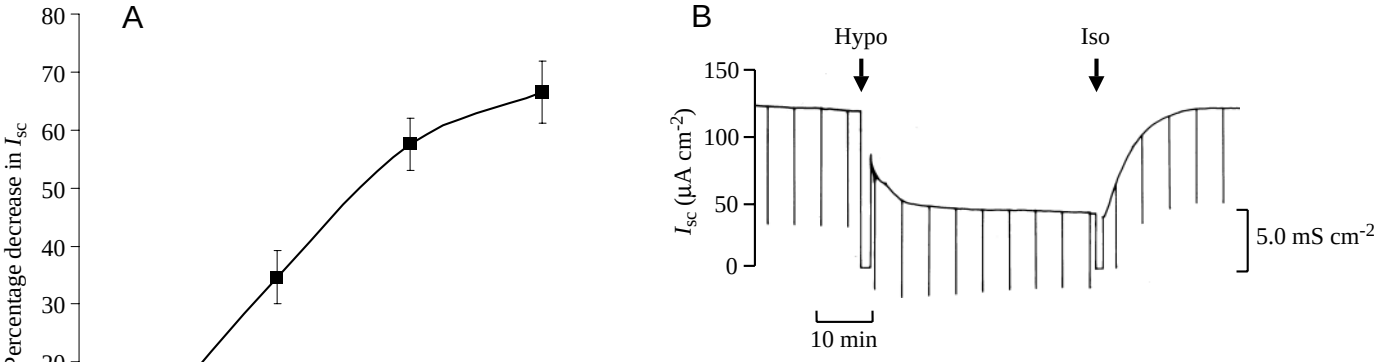

Fig. 2. (A) Inhibition of Cl^- secretion, measured as short-circuit current (I_{sc}), across mummichog opercular epithelium *in vitro* (N=10) over 40 min with no change in the bathing solutions (0; flow-through of iso-osmotic bathing solution) or a stepwise reduction in serosal osmolality by flow-through of progressively more dilute bathing solution. Increased hypo-osmotic shock evoked progressively larger reductions in I_{sc}. Values are means ± S.E.M. (B) An example showing the time course of I_{sc} inhibition by hypotonic shock (Hypo; 60 mosmol kg^{-1} decrease by flow-through of diluted Cortland's saline) applied to the basolateral side of an isolated opercular epithelium. The tissue was unclamped during the solution changes. The transients are current responses to a command change in clamp voltage from 0 to –10 mV and represent transepithelial conductance G_t (scale bar at right), which decreased along with the I_{sc}. The effect was freely reversible on reintroduction of full-strength saline (Iso).

of 20 mosmol kg^{-1} significantly decreased Cl^- secretion rate (measured as I_{sc}) by approximately 25 % compared with control flow-through of iso-osmotic bathing solution (Fig. 2A). Further decreases of 40 and 60 mosmol kg^{-1} inhibited I_{sc} further. A decrease of 60 mosmol kg^{-1} reduced I_{sc} by approximately 65 %. The example shown in Fig. 2B demonstrates the reversibility of the hypo-osmotic effect when flow-through of dilute Cortland's saline (Hypo; a step change of 60 mosmol kg^{-1}) was followed by flow-through of full-strength saline (Iso).

To ensure that the effect was not the result of a reduced concentration of Na^+ and Cl^- on the basolateral side, paired membranes were exposed either to iso-osmotic/iso-ionic Cortland's saline or to an iso-osmotic/hypo-ionic Cortland's saline with reduced [NaCl] but supplemented with mannitol (Fig. 3). A reduction in [NaCl] from 160 to 120 mmol l^{-1} with mannitol added to maintain osmolality had no effect on I_{sc} (Fig. 3). However, hypotonic shock given after this pretreatment (by flow-through of the diluted bathing solution for the control and test, diluted to 75 % of normal) produced significant reductions in I_{sc} that were the same for the mannitol-supplemented and control membranes. Hence, the reduction in I_{sc} is connected to the osmolality of the serosal bathing solution and not to similar changes in NaCl content.

The reduction in I_{sc} was reversible by reintroduction of isotonic solutions. In a separate group of membranes, I_{sc} before hypotonic shock was 183.3±11.9 μA cm^{-2} (N=6), decreased after hypotonic shock to 112.1±9.3 μA cm^{-2} (P<0.001 compared with controls) and recovered to 85 % of the original current 155.8±10.7 μA cm^{-2} when isotonic solutions were reintroduced. The decrease in I_{sc} was accompanied by a significant decrease in transepithelial conductance G_t, in this case from 9.77±0.7 to

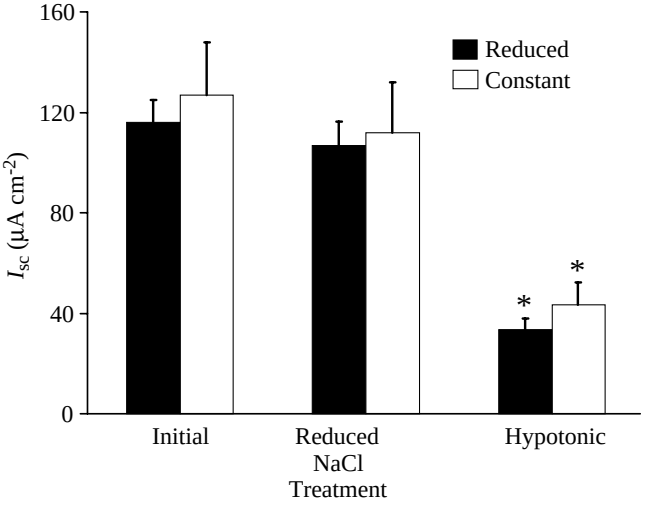

Fig. 3. Reduction in NaCl content of the serosal bathing solution in the presence of mannitol to maintain a constant osmolality (filled columns; treatment) had no effect on short-circuit current (I_{sc}) compared with control membranes (open columns; treatment), indicating that the effect is osmotic, not ionic. Subsequent hypotonic shock (serosal bathing solution, a reduction of 71 mosmol kg^{-1}) inhibited I_{sc} equally for control and low-NaCl-treated membranes (*P<0.01, paired t-test compared with treatment period, N=7). Values are means + S.E.M.

6.41±0.47 mS cm^{-2} (P<0.002, paired t-test N=6), which recovered to 92 % of initial levels (8.97±0.52 mS cm^{-2}) when isotonic solutions were reintroduced. There was no significant change in V_t after hypotonic shock; V_t was 18.9±0.96 mV initially, 17.98±1.88 mV (P>0.70, paired t-test compared with

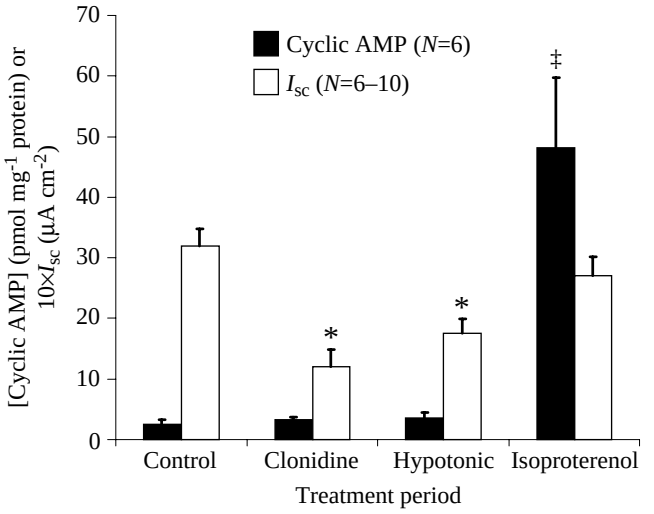

Fig. 4. Radioimmunoassay of cyclic AMP and short-circuit current (I_{sc}) of opercular epithelial membranes *in vitro*. Under control conditions (isotonic solutions on both sides), after addition of the α_2-adrenergic agonist clonidine (1.0 μmol l^{-1}, serosal side), after basolateral hypotonic shock and after addition of the β-adrenergic agonist isoproterenol (1.0 μmol l^{-1}) as a positive control. Hypotonic shock and clonidine had no effect on resting cyclic AMP levels, but inhibited I_{sc} significantly (*P<0.01, unpaired *t*-test compared with control levels; N=6–10), while isoproterenol significantly elevated cyclic AMP levels (‡P<0.01, unpaired *t*-test compared with controls) and had little effect on I_{sc}. Values are means + S.E.M.

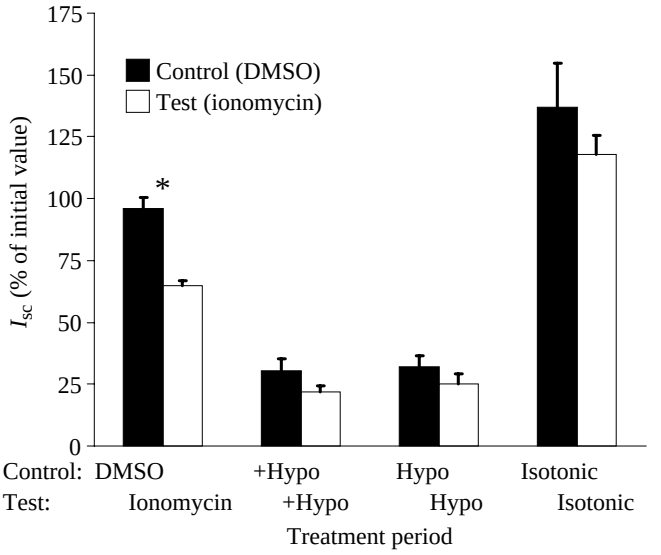

Fig. 5. Measurements of short-circuit current (I_{sc}) during sequential treatment of paired opercular membranes (N=8) with the Ca^{2+} ionophore ionomycin (1.0 μmol l^{-1}) and with hypotonic shock (Hypo). Test membranes (open columns) were compared with the control membranes that received dimethylsulphoxide (DMSO) only (filled columns). DMSO had no effect on I_{sc}, while ionomycin significantly inhibited I_{sc} (*P<0.01 compared with control; left-hand pair of histograms). Hypotonic shock inhibited I_{sc} approximately equally in ionomycin- and DMSO-treated membranes (central second and third pairs of histograms). Restoration of isotonic bathing solutions produced full recovery of I_{sc} with a slight overshoot, with no significant difference between DMSO- and ionomycin-treated membranes (right-hand pair of histograms). Values are means + S.E.M.

the control period, N=6) after hypotonic shock and 17.42±0.81 mV (P>0.05 compared with control period) on return to isotonic bathing solutions.

Cyclic AMP mediation

Assays of cyclic AMP after hypotonic shock indicated no change in cyclic AMP content compared with controls or with tissues exposed to the α_2-adrenergic agonist clonidine. Both these treatments reduced Cl^- secretion rate (measured as I_{sc}; Fig. 4). As a positive control, the β-adrenergic agonist isoproterenol increased cyclic AMP content approximately 14-fold, accompanied by an increase in I_{sc} compared with the clonidine and hypotonic pretreatments. Forskolin (10 μmol l^{-1}) by itself increased cyclic AMP content to 362.2±43.8 pmol mg^{-1} protein (N=6), which did not differ (P>0.5, unpaired *t*-test) from the value for membranes treated with forskolin plus hypotonic shock (389.1± 34.2 pmol mg^{-1} protein; N=6); hence, hypotonic shock does not reduce resting or forskolin-stimulated levels of cyclic AMP in opercular epithelia.

Ca^{2+} mediation

To test for possible mediation of the hypotonic effect by intracellular Ca^{2+}, the Ca^{2+} ionophore ionomycin (1.0 μmol l^{-1}) was added followed by hypotonic shock and compared with a parallel running control membrane from the same animal (Fig. 5). DMSO had no detectable effect, while ionomycin reduced I_{sc} to 65±3.9 %. Hypotonic shock applied after ionomycin, however, produced a significant reduction in I_{sc} (Fig. 5), and the percentage reduction was similar to that produced in control membranes. Subsequent restoration of normal osmolality produced a rebound increase in I_{sc} both in ionomycin-treated membranes and in controls (Fig. 5).

Disruption of Ca^{2+} responses was also attempted in a low-[Ca^{2+}] regime (100 μmol l^{-1} Ca^{2+} on the apical side and approximately 1.0 μmol l^{-1} on the serosal side) with and without added thapsigargin (1.0 μmol l^{-1}) to deplete intracellular pools of Ca^{2+}. The low-[Ca^{2+}] regime by itself had no effect on I_{sc} (Fig. 6), indicating that the integrity of tight junctions was maintained and that the rate of ion secretion was unaffected. An hour of exposure to thapsigargin and the DMSO vehicle had little effect on I_{sc}; in both cases, there was a non-significant reduction of 10–15 % from the starting I_{sc}. Basolateral hypotonic shock inhibited I_{sc} in control and thapsigargin-treated membranes to the same extent (45 % for the thapsigargin-treated membranes and 49 % for the DMSO control), and there was no significant difference between the control and treated membranes in responsiveness to hypotonic shock.

Protein tyrosine kinase mediation

The protein tyrosine kinase inhibitor genistein

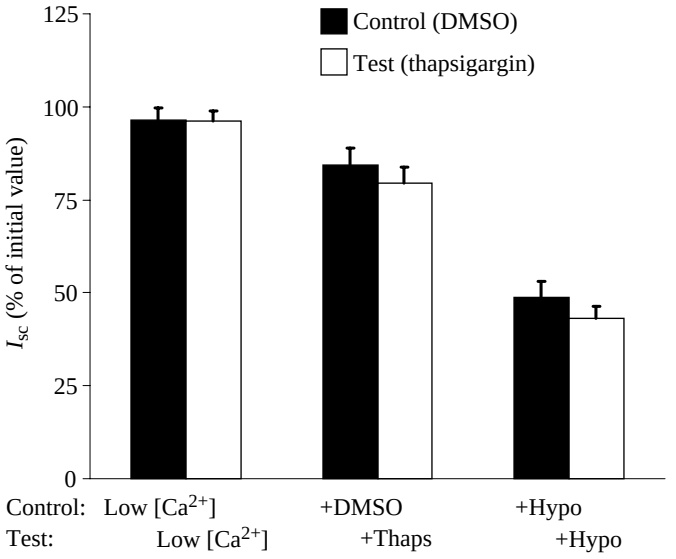

Fig. 6. Depletion of Ca^{2+} in opercular epithelia (N=7) using low-$[Ca^{2+}]$ bathing solutions (<1.0 μmol l^{-1} serosal Ca^{2+}, 0.1 mmol l^{-1} mucosal Ca^{2+}) with and without added thapsigargin (Thaps; 1.0 μmol l^{-1}, 1 h) had little effect on short-circuit current (I_{sc}) (compare the left-hand and central pair of histograms), and subsequent hypotonic shock (Hypo) produced a significant (P<0.001) inhibition of approximately 50 % of the remaining I_{sc} (right-hand pair of histograms). Values are means ± S.E.M.

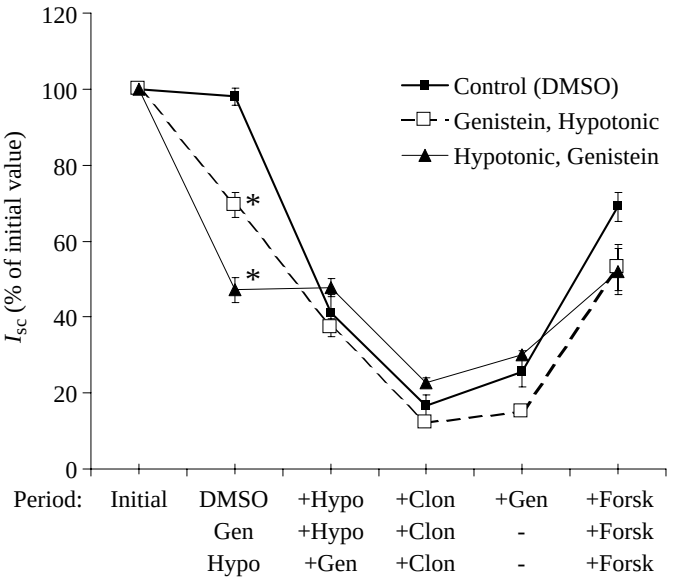

Fig. 7. A comparison of the effects of genistein (Gen) and hypotonicity (Hypo) on the inhibition of Cl^- secretion by opercular epithelia (N=6). In the first treatment period, the dimethylsulphoxide (DMSO) vehicle had no effect, genistein reduced short-circuit current (I_{sc}) significantly by approximately 30 % and hypotonicity alone reduced I_{sc} by approximately 55 % (*P<0.001 compared with the control treatment). The second treatment period demonstrates that hypotonic shock to the genistein-treated membranes brought the current to the same level as for hypotonic treatment alone. Subsequent addition of clonidine inhibited I_{sc} and was equally effectively for all treatments. Addition of genistein to the hypotonic clonidine-treated membranes produced a small, but statistically significant (P<0.05), rise in I_{sc} (fourth period compared with third period). Finally, forskolin (Forsk; 10 μmol l^{-1}) significantly stimulated I_{sc} in all groups (fifth period). I_{sc} was allowed to come to a new steady state after each treatment (approximately 30–40 min). Values are means ± S.E.M.

(100 μmol l^{-1}, basolateral side) was applied to membranes in isotonic and hypotonic conditions. Genistein in isotonic conditions significantly inhibited I_{sc} by 31 % (P<0.001 compared with the DMSO control), while the DMSO control did not change (Fig. 7). Subsequent hypo-osmotic shock inhibited I_{sc} further to 63 % of the initial I_{sc}. Hypotonic shock inhibited I_{sc} by 54 % (P<0.005 compared with genistein), but there was no additional effect with the subsequent addition of genistein (100 μmol l^{-1}, serosal side). The net effect was that hypotonic shock before or after genistein treatment produced approximately the same total inhibition of I_{sc}, i.e. the effects of hypotonic shock and genistein were not additive. Genistein did not block the inhibition of I_{sc} by the α_2-adrenergic agonist clonidine (1.0 μmol l^{-1}, serosal side), with clonidine producing more than 80 % inhibition compared with the initial I_{sc}. Interestingly, when genistein was added to the DMSO control membranes after clonidine inhibition, there was a small but statistically significant increase in I_{sc} from 35.4±15.6 to 53.7±19.4 μA cm^{-2} (P<0.01, paired t-test). Finally, addition of forskolin (10 μmol l^{-1}, basolateral side) significantly increased I_{sc} in all three groups to approximately the same extent (Fig. 7).

Daidzein is a phytoestrogen closely related to genistein, differing only at carbon 7 in having an additional hydroxyl group. It is used as a control substance for the action of genistein on protein tyrosine kinase and is inactive in this respect (Lavens et al., 1992). Genistein, but not daidzein, inhibited I_{sc} in a separate set of membranes (Fig. 8), but neither drug blocked the subsequent inhibition of I_{sc} by clonidine or stimulation of I_{sc} by forskolin.

Discussion

Hypotonic control of ion secretion

When mummichogs are transferred directly from sea water to fresh water there is a decrease in plasma $[Na^+]$ (Fig. 1) and in osmolality, the decrease averaging 29 % at 6 h after transfer. Hypotonic shock applied to the basolateral side of the isolated opercular epithelium, to mimic this decrease in plasma osmolality, reversibly (see Results and Fig. 5) inhibited ion secretion in a dose-dependent fashion with the maximal effect being approximately 70 % inhibition of I_{sc} (Fig. 2A). The effect is osmotic because a similar reduction in the NaCl content of the bathing solution but with mannitol added to maintain osmolality eliminated the effect (Fig. 3).

Plasma osmolality changes in euryhaline species have been observed for many years, but they have only recently been connected with the control of rates of transport. Zadunaisky et

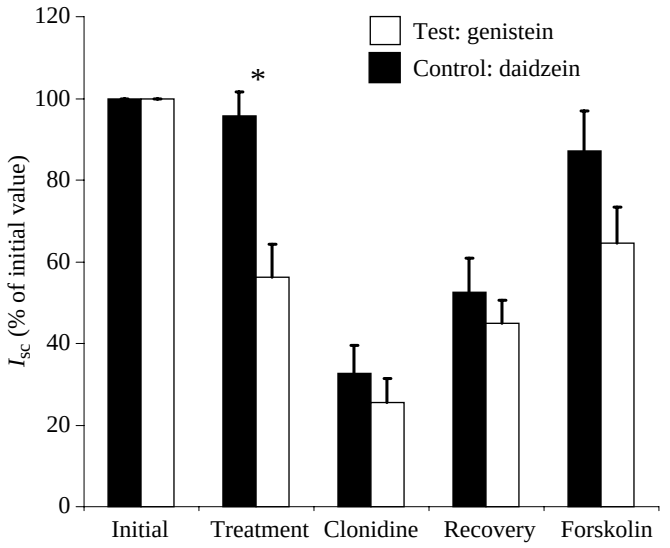

Fig. 8. Addition of 100 μmol l^{-1} daidzein (filled columns) had no effect on short-circuit current (I_{sc}), while 100 μmol l^{-1} genistein (open columns) decreased I_{sc} by approximately 40 % (P<0.01, unpaired t-test compared with daidzein treatment, N=7). The effects of subsequent additions of clonidine and forskolin to genistein-treated and daidzein-treated (control) membranes were not significantly different. I_{sc} was allowed to come to a new steady state after each treatment (approximately 30–40 min). Values are means + S.E.M.

al. (1995) observed significant increases in the I_{sc} of mummichog opercular epithelium when hypertonic bathing solutions were applied to the basolateral side. Similar hypertonicity of the apical bath was without effect. Since then, preliminary reports have suggested that the reverse effect occurs in response to hypotonic bathing solutions (Zadunaisky et al., 1997). When mummichogs are transferred directly from fresh water to sea water, we found that our normal saline was in fact hypotonic (because the plasma osmolality rises after transfer), and the full transport capacity was observed only when the osmolality of the bathing solution was matched to the measured osmolality of the plasma at that particular stage in the acclimation (Marshall et al., 1999). The fact that these osmotic effects can be evoked *in vitro* is a demonstration that they are not mediated by neural or hormonal signals and are probably direct effects on individual cells or possibly parahormonal effects within the tissue. However, two common parahormonal effectors, ATP and adenosine, have no effect on isolated opercular epithelial transport (W. S. Marshall, unpublished observations). The hypotonic effect does not completely eliminate the I_{sc}, but *in vivo* Cl^- secretion stops completely, so it is likely that there are additive effects with other regulatory systems. The inhibition of Cl^- secretion by the α_2-agonist clonidine was unaffected by hypotonic shock (Figs 7, 8); hence, this independent adrenergic action could be additive to the osmotic response. The adrenergic inhibition is probably neural (autonomic) rather than hormonal because firing of the autonomic nerves innervating the tissue produces substantial inhibition by α_2-adrenoceptors (Marshall et al., 1998c). The combined action of adrenergic stimulation and direct hypotonic shock effects could account for the full elimination of Cl^- secretion within an hour of exposure to fresh water.

The reduction in I_{sc} is accompanied by a decrease in G_t but little, if any, change in V_t (see Results), suggesting that the inhibition is caused primarily by a reduction in the activity of a conductive pathway in the epithelium. This could represent a shutdown of either the apically located CFTR anion channel (Marshall et al., 1995; Singer et al., 1998) or the basolaterally located $Na^+/K^+/2Cl^-$ cotransporter. There is a preliminary report of large changes, with salinity acclimation of mummichogs, in the absolute amount and degree of phosphorylation of the $Na^+/K^+/2Cl^-$ cotransporter in the gills (Flemmer and Forbusch, 1999). In addition, CFTR activity is known to vary with its level of phosphorylation (Gadsby and Nairn, 1999) and with membrane trafficking of CFTR to the plasma membrane (Lehrich et al., 1998). At present, it is not clear which transporter is affected by hypotonic shock.

In fully adapted freshwater mummichog, the opercular epithelium does not secrete ions; instead, there is evidence for active Cl^- uptake (Marshall et al., 1998b). In whole-animal flux experiments with freshwater-adapted mummichog, rates of Cl^- uptake appear to be low while rates of Na^+ uptake are greater, and the affinity for the uptake of both ions is low compared with that of freshwater species (Patrick et al., 1997). While the mechanisms involved in freshwater acclimation remain to be resolved for mummichog, it is clear that elimination of Cl^- secretion is a major early response during freshwater acclimation and that this decrease is mediated in part by plasma hypotonicity.

In a related paper (K. Daborn and W. S. Marshall, in preparation), data are presented to implicate the active closure of apical crypts by pavement cells, an effect initiated by hypotonic shock and mediated by actin microfilaments that may account for the reduction in passive ion loss by the opercular epithelium shortly after transfer from sea water to fresh water. In this combination of events, both active ion secretion and diffusional ion loss are minimized to the benefit of the animal in its hypotonic environment.

Mediation of the osmotic response

Protein tyrosine kinase

Genistein is a well-known inhibitor of protein tyrosine kinase (Lavens et al., 1992). When added to the basolateral side of the opercular epithelium, it inhibited I_{sc} in a manner that was not additive to the hypotonic response, suggesting that the hypotonic response involves inhibition of tyrosine kinase (Fig. 7). The inhibition of I_{sc} was not evoked by an analogue of genistein, daidzein (Fig. 8), which does not inhibit protein tyrosine kinase (Lavens et al., 1992).

Because genistein has some unrelated actions, these must also be considered. Genistein, but not daidzein, binds to and directly activates CFTR anion channels in a manner additive to cyclic AMP and protein kinase A (PKA) activation of CFTR (Illek et al., 1996; Hwang et al., 1997; Weinreich et al., 1997;

for a review, see Gadsby and Nairn, 1999). The action may be associated with inhibition of serine/threonine protein phosphatase attached to CFTR (Reenstra et al., 1996). This effect puts into question reports of stimulation of rates of Cl^- transport by genistein that were attributed to inhibition of protein tyrosine kinase (Illek et al., 1995; Lehrich and Forrest, 1995; Lehrich et al., 1998; Shuba et al., 1996). Recently, genistein has also been reported to inhibit cyclic AMP phosphodiesterase in a neural cell line (Nichols and Morimoto, 1998), an effect that would also tend to activate cyclic-AMP-mediated stimulatory pathways. Genistein does have stimulatory effects on opercular epithelia as well, but only if the ion transport has been previously inhibited. Of interest here is the observation that genistein inhibited Cl^- secretion, and did not stimulate it.

The effect of genistein in the mummichog opercular epithelium is probably an action involving inhibition of protein tyrosine kinase, rather than other reported actions. The action of genistein in untreated membranes is an inhibition of Cl^- secretion, not a stimulation, as seen in other anion-secreting systems (Illek et al., 1996; Weinreich et al., 1997; Zhou et al., 1998), so direct activation of CFTR is not the action in teleosts. Also, genistein was added basolaterally, not on the apical side where the drug could have the required access to the extracellular side of CFTR for CFTR stimulation (Zhou et al., 1998). Genistein is an activator of Ca^{2+}-dependent K^+ channels in vascular smooth muscle (Xiong et al., 1995), but in the opercular epithelium Ca^{2+} mediation (e.g. by the Ca^{2+} ionophore ionomycin; Marshall et al., 1993) produces not activation of K^+ channels (which would increase Cl^- secretion) but rather inhibition of Cl^- secretion. Finally, daidzein, an inactive analogue of genistein that does not inhibit protein tyrosine kinase (Lavens et al., 1992), was inactive in this preparation. To summarize, genistein appears to act in the opercular epithelium by inhibiting protein tyrosine kinase, possibly by reducing tyrosine phosphorylation of CFTR and thus inhibiting Cl^- secretion. This conclusion is supported by the reported activation of CFTR in mouse fibroblasts by tyrosine kinase p60c-src (Fischer and Machen, 1996).

Lack of involvement of cyclic AMP

Hypo-osmotic shock did not affect resting or forskolin-stimulated cyclic AMP levels in opercular epithelia (Fig. 4), so it is unlikely that the osmotic response is mediated by changes in cyclic AMP levels or the actions of protein kinase A. However, agents that increase cyclic AMP levels (forskolin and isoproterenol) can override hypotonic inhibition (Figs 4, 7), so the effects of hypotonic shock would seem to be independent of cyclic-AMP-mediated events. The lack of effect of clonidine on cyclic AMP levels is consistent with the action of α_2-adrenoceptors in this system, which inhibit ion transport *via* an increase in intracellular $[Ca^{2+}]$ (Marshall et al., 1993), and the release of Ca^{2+} appears to be *via* elevation of inositol trisphosphate levels and, presumably, phospholipase C (Marshall et al., 1998c). Similar α_2-adrenergic actions have been reported in another epithelial system, the mammalian distal convoluted tubule (Gesek, 1996).

Lack of involvement of intracellular Ca^{2+}

The Ca^{2+} ionophore ionomycin (but not A23187) inhibited Cl^- secretion, and Ca^{2+} apparently contributed to the inhibition through α_2-adrenoceptors in the epithelium (Marshall et al., 1993). We therefore tested Ca^{2+} mediation of the hypotonic response. Mediation of the hypotonic response by intracellular Ca^{2+} is unlikely because flooding the cells in the membrane with Ca^{2+} using ionomycin had little effect on the response (Fig. 5). In addition, Ca^{2+} depletion on the basolateral side by itself or in combination with depletion of intracellular Ca^{2+} stores using thapsigargin (Fig. 6) failed to block the hypotonic effect.

Volume regulation and hypotonicity

The relationship between the inhibition of transmural transport described here and cell volume regulation in chloride cells is unknown. It is likely that chloride cells undergo a regulatory volume decrease in response to hypotonic shock; such responses characteristically include activation of KCl transport that is phosphorylation-dependent (Berenbrink et al., 1997; for a review, see Cossins and Gibson, 1997). Efflux of KCl and organic osmolytes such as taurine carries fluid out of the cell osmotically and restores cell volume. Many cells employ a volume-sensitive outwardly rectifying anion channel as part of the regulatory volume decrease (for a review, see Okada, 1997); however, this channel does not appear to be present in mummichog chloride cells (Marshall et al., 1995). The volume regulatory response of chloride cells, which probably involves K^+, Cl^- and organic osmolytes exiting the cell across the basolateral membrane, will be an interesting area for future investigation.

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References

Berenbrink, M., Weaver, Y. R. and Cossins, A. R. (1997). Defining the volume dependence of multiple K flux pathways of trout red blood cells. *Am. J. Physiol.* **272**, C1099–C1111.

Burg, M. B., Kwon, E. D. and Kültz, D. (1997). Regulation of gene expression by hypertonicity. *Annu. Rev. Physiol.* **59**, 437–455.

Cossins, A. R. and Gibson, J. S. (1997). Volume-sensitive transport systems and volume homeostasis in vertebrate red blood cells. *J. Exp. Biol.* **200**, 343–352.

Fischer, H. and Machen, T. E. (1996). The tyrosine kinase p60 c-SRC regulates the fast rate of the cystic fibrosis transmembrane conductance regulator chloride channel. *Biophys. J.* **71**, 3073–3082.

Flemmer, A. W. and Forbush III, B. (1999). Changes in amount and phosphorylation state of the Na^+–K^+–Cl^- cotransporter (NKCC) in teleost gill during salt adaptation. *FASEB J.* **13**, A399 (Abstract).

Gadsby, D. C. and Nairn, A. C. (1999). Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiol. Rev.* **79**, S77–S107.

Gesek, F. A. (1996). α_2-Adrenergic receptors activate phospholipase C in renal epithelial cells. *Molec. Pharmac.* **50**, 407–414.

Hwang, T.-C., Wang, F., Yang, I. C.-H. and Reenstra, W. W. (1997). Genistein potentiates wild-type and ΔF508-CFTR channel activity. *Am. J. Physiol.* **273**, C988–C998.

Illek, B., Fischer, H. and Machen, T. E. (1996). Alternate stimulation of apical CFTR by genistein in epithelia. *Am. J. Physiol*. **270**, C265–C275.

Illek, B., Fischer, H., Santos, G. F., Widdicombe, J. H., Machen, T. E. and Reenstra, W. W. (1995). cAMP-independent activation of CFTR Cl^- channels by the tyrosine kinase inhibitor genistein. *Am. J. Physiol.* **268**, C886–C893.

Jacob, W. F. and Taylor, M. H. (1983). The time course of seawater acclimation in *Fundulus heteroclitus* L. *J. Exp. Zool.* **228**, 33–39.

Lavens, S. E., Peachell, P. T. and Warner, J. A. (1992). Role of tyrosine kinases in IgE-mediated signal transduction in human lung mast cells and basophils. *Am. J. Respir. Cell Mol. Biol*. **7**, 637–644.

Lehrich, R. W., Aller, S. G., Webster, P., Marino, C. R. and Forrest, J. N., Jr (1998). Vasoactive intestinal peptide, forskolin and genistein increase apical CFTR trafficking in the rectal gland of the spiny dogfish, *Squalus acanthias*. *J. Clin. Invest.* **101**, 737–745.

Lehrich, R. W. and Forrest, J. N., Jr (1995). Tyrosine phosphorylation is a novel pathway for regulation of chloride secretion in shark rectal gland. *Am. J. Physiol.* **269**, F594–F600.

Maetz, J. (1974). Aspects of adaptation to hypo-and hyper-osmotic environments. In *Biochemical and Biophysical Perspectives in Marine Biology*, vol. 1 (ed. D. C. Malins and J. R. Sargeant), pp. 1–167. Academic Press, London.

Marshall, W. S. and Bryson, S. E. (1997). Genistein inhibits Cl^- secretion by teleostean chloride cells, a novel CFTR containing epithelial system. *FASEB J.* **11**, A305 (Abstract).

Marshall, W. S. and Bryson, S. E. (1998). Transport mechanisms of seawater chloride cells: An inclusive model of a multifunctional cell. *Comp. Biochem. Physiol.* **119**A, 97–106.

Marshall, W. S., Bryson, S. E., Darling, P., Whitten, C., Patrick, M., Wilkie, M., Wood, C. M. and Buckland-Nicks, J. (1998a). NaCl transport and ultrastructure of opercular epithelium from a freshwater-adapted euryhaline teleost, *Fundulus heteroclitus. J. Exp. Zool.* **277**, 23–37.

Marshall, W. S., Bryson, S. E. and Garg, D. (1993). α_2-Adrenergic inhibition of Cl^- transport by opercular epithelium is mediated by intracellular Ca^{2+}. *Proc. Natl. Acad. Sci. USA* **90**, 5504–5508.

Marshall, W. S., Bryson, S. E. and Luby, T. (1998b). Control of epithelial Cl^- secretion by basolateral osmolality in the euryhaline teleost *Fundulus heteroclitus*. *FASEB. J.* **12**, A122 (Abstract).

Marshall, W. S., Bryson, S. E., Midelfart, A. and Hamilton, W. F. (1995). Low conductance anion channel activated by cyclic AMP in teleost Cl-secreting cells. *Am. J. Physiol.* **268**, R963–R969.

Marshall, W. S., DuQuesnay, R. M., Gillis, J. M., Bryson, S. E. and Liedtke, C. M. (1998c). Neural modulation of salt secretion in teleost opercular epithelium by α_2-adrenergic receptors and inositol 1,4,5-trisphosphate. *J. Exp. Biol.* **201**, 1959–1965.

Marshall, W. S., Emberley, T. R., Singer, T. D., Bryson, S. E. and McCormick, S. D. (1999). Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. *J. Exp. Biol.* **202**, 1535–1544.

Motais, R., Garcia-Romeu, F. and Maetz, J. (1966). Exchange diffusion effect and euryhalinity in teleosts. *J. Gen. Physiol.* **50**, 391–422.

Nichols, M. R. and Morimoto, B. H. (1998). Genistein directly inhibits cyclic-AMP specific phosphodiesterase (PDE4) activity. *FASEB J.* **12**, A1483 (Abstract).

Okada, Y. (1997). Volume expansion-sensing outward rectifier Cl^- channel: fresh start to the molecular identity and volume sensor. *Am. J. Physiol.* **273**, C755–C789.

Patrick, M. L., Pärt, P., Marshall, W. S. and Wood, C. M. (1997). Characterization of ion and acid–base transport in the fresh water adapted mummichog (*Fundulus heteroclitus*). *J. Exp. Biol.* **279**, 208–219.

Potts, W. T. W. and Evans, D. H. (1967). Sodium and chloride balance in the killifish *Fundulus heteroclitus. Biol. Bull.* **131**, 411–425.

Reenstra, W. W., Yurku-Mauro, K., Dam, A., Ramen, S. and Shorten, S. (1996). CFTR chloride channel activation by genistein: the role of serine/threonine protein phosphatases. *Am. J. Physiol.* **271**, C650–C657.

Shuba, L. M., Asai, T., Pelzer, S. and McDonald, T. F. (1996). Activation of cardiac chloride conductance by tyrosine kinase inhibitor, genistein. *Br. J. Pharmac.* **119**, 335–345.

Singer, T. D., Tucker, S. J., Marshall, W. S. and Higgins, C. F. (1998). A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F. heteroclitus*. *Am. J. Physiol.* **274**, C715–C723.

Weinreich, F., Wood, P. G., Riordan, J. R. and Nagel, G. (1997). Direct action of genistein on CFTR. *Pflügers Arch.* **434**, 484–491.

Wood, C. M. and Marshall, W. S. (1994). Ion balance, acid–base regulation and chloride cell function in the common killifish, *Fundulus heteroclitus* – A euryhaline estuarine teleost. *Estuaries* **17**, 34–52.

Xiong, Z., Burnette, E. and Cheung, D. W. (1995). Modulation of Ca^{2+}-activated K^+ channel activity by tyrosine kinase inhibitors in vascular smooth muscle cell. *Eur. J. Pharmac.* **290**, 117–123.

Zadunaisky, J. A., Balla, M. and Colon, D. E. (1997). A reduction in chloride secretion by lowered osmolarity in chloride cells of *Fundulus heteroclitus*. *Bull. Mt Desert Island Biol. Lab.* **24**, 52 (Abstract).

Zadunaisky, J. A., Cardona, S., Au, L., Roberts, D. M., Fisher, E., Lowenstein, B., Cragoe, E. J. and Spring, K. R. (1995). Chloride transport activation by plasma osmolarity during rapid adaptation to high salinity of *Fundulus heteroclitus. J. Membr. Biol.* **143**, 207–217.

Zhou, S. S., Hazama, A. and Okada, Y. (1998). Tyrosine kinase independent extracellular action of genistein on the CFTR Cl^- channel in guinea pig ventricular myocytes and CFTR-transfected mouse fibroblasts. *Jap. J. Physiol.* **48**, 389–396.