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Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1 and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium

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Summary

Hypotonic shock rapidly inhibits Cl^- secretion by chloride cells, an effect that is osmotic and not produced by NaCl-depleted isosmotic solutions, yet the mechanism for the inhibition and its recovery are not known. We exposed isolated opercular epithelia, mounted in Ussing chambers, to hypotonic shock in the presence of a variety of chemicals: a general protein kinase C (PKC) inhibitor chelerythrine, Gö6976 that selectively blocks PKCα and β subtypes, H-89 that blocks PKA, SB203580 that blocks p38 mitogen-activated protein kinase (MAPK), as well as serine/threonine protein phosphatase (PP1 and 2A) inhibitor okadaic acid, and finally tamoxifen, a blocker of volume-activated anion channels (VSOAC). Chelerythrine has no effect on hypotonic inhibition but blocked the recovery, indicating PKC involvement in stimulation. Gö6976 had little effect, suggesting that PKCα and PKCβ subtypes are not involved. H-89 did not block hypotonic inhibition but decreased the recovery, indicating PKA may be involved in the recovery and overshoot (after restoration of isotonic conditions). SB203580 significantly enhanced the decrease in current by hypotonic shock, suggesting an inhibitory role of p38 MAPK in the hypotonic inhibition. Okadaic acid increased the steady state current, slowed the hypotonic inhibition but made the decrease in current larger; also the recovery and overshoot were completely blocked. Hypotonic stress rapidly and transiently increased phosphorylated p38 MAPK (pp38) MAPK (measured by western analysis) by eightfold at 5 min, then more slowly again to sevenfold at 60 min. Hypertonic shock slowly increased p38 by sevenfold at 60 min. Phosphorylated JNK kinase was increased by 40–50% by both hypotonic and hypertonic shock and was still elevated at 30 min in hypertonic medium. By immunoblot analysis it was found that the stress protein kinase (SPAK) and oxidation stress response kinase 1 (OSR1) were present in salt and freshwater acclimated fish with higher expression in freshwater. By immunocytochemistry, SPAK, OSR1 and phosphorylated focal adhesion kinase (pFAK) were colocalized with NKCC at the basolateral membrane. The protein tyrosine kinase inhibitor genistein (100 $\mu mol\ l^{-1}$) inhibited Cl^- secretion that was high, increased Cl^- secretion that was low and reduced immunocytochemical staining for phosphorylated FAK. We present a model for rapid control of CFTR and NKCC in chloride cells that includes: (1) activation of NKCC and CFTR *via* cAMP/PKA, (2) activation of NKCC by PKC, myosin light chain kinase (MLCK), p38, OSR1 and SPAK, (3) deactivation of NKCC by hypotonic cell swelling, Ca^{2+} and an as yet unidentified protein phosphatase and (4) involvement of protein tyrosine kinase (PTK) acting on FAK to set levels of NKCC activity.

Key words: *Fundulus heteroclitus*, protein kinase, protein phosphatase, SB203580, regulatory volume decrease, okadaic acid, chelerythrine, NKCC1, Na^+,K^+,$2Cl^-$ cotransport, gill epithelium.

Introduction

Osmosensing by transporting epithelial cells is an intriguing way to regulate transepithelial transport, because it does not involve hormones or neurotransmitters. Hypotonic shock rapidly inhibits Cl^- secretion by chloride cells, an effect that is osmotic and not produced by NaCl-depleted isosmotic solutions (Zadunaisky et al., 1997; Marshall et al., 2000; Hoffmann et al., 2002). It is well known from various cells including Ehrlich ascites tumour cells, that cell shrinkage followed by a regulatory volume increase take place, when cells that have seen a hypotonic medium for some time are taken back to an isotonic medium, a phenomenon called regulatory volume increase after regulatory volume decrease (RVD-after-RVI protocol) (Hoffmann and Simonsen, 1989). It is moreover well know for the operculum epithelium that such cell shrinkage evokes large current increases (Zadunaisky et al., 1995; Hoffman et al., 2002). Osmotic changes brought on

in whole animals by salinity transfer are associated with changes in gill tissue MAP kinase expression and phosphorylation (Kültz and Avila, 2001), pointing to the involvement of kinase cascades in volume responses by these ion transporting cells.

Hypotonic shock is physiologically relevant because small estuarine fish such as the common killifish (mummichog), *Fundulus heteroclitus*, forage in shallow water following advancing tides and are exposed regularly to freshwater (FW) microenvironments. Typically the salinity in these shallows is low, ~3% seawater (1.0 g l^{-1}) or lower. Because the animals return to high salinity between tides, only temporary 'coping' mechanisms are required, not permanent acclimation to FW (Marshall, 2003). In the first few hours, blood osmolality and ion content are reduced, producing a hyposmotic cue to ion transporting cells (Marshall et al., 2000). Hypotonic shock in vitro rapidly inhibits the ion secretion by mitochondria-rich chloride secreting cells of the gill and opercular epithelium (Zadunaisky et al., 1998; Marshall et al., 2000), an effect mimicked by the protein tyrosine kinase inhibitor genistein (Marshall et al., 2000). Surprisingly, the hypotonic inhibition was also exacerbated by the phosphatase inhibitor calyculin A (Zadunaisky et al., 1998).

Killifish opercular epithelium and related teleost membranes are model systems containing mitochondria-rich cells used to study the regulation of salt transport. Reduction in ion transport after transfer to freshwater includes an inhibition of active Cl^- secretion and passive diffusive ion loss in a three-stage process spanning approx. 30 min. There is a combination of sympathetic neural reflex mediated by α_2-adrenoceptors operating *via* intracellular inositol trisphosphate through intracellular Ca^{2+} (Marshall et al., 1993, 1998), a rapid cellular hypotonic shock response (Marshall et al., 2000) and finally a covering over of ion secreting cells by pavement cells (Daborn et al., 2001). These three steps effectively minimize salt loss in dilute media. The upregulation of salt secretion on return to full strength seawater (SW) may be *via* hormones (arginine vasotocin and urotensin I) and a neurotransmitter (vasoactive intestinal polypeptide) in combination with hypertonic shock (Hoffmann et al., 2002). Because in nature the rapid inhibition of Cl^- secretion is short lived (a few hours), voluntary (involving shoaling behaviour) and mediated by autonomic reflex and neurohormones in combination with direct effects of blood tonicity on ion transporting cells, the concept of a nonstressful salinity change is put forward.

We hypothesized that the basolateral Na^+,K^+,2Cl^- cotransporter, NKCC1 isoform, which is the mechanism for Cl^- entry across the basolateral membrane by secondary active transport, was the focal point of the regulation. Cl^- secretion by the isolated opercular membrane of killifish is sensitive to serosal bumetanide, piretanide and furosemide (EC_{50} of 40, 52 and 295 μmol l^{-1}, respectively) and insensitive to DIDS and thiazide type drugs, indicating an operational NKCC-type cotransporter on the basolateral membrane (Eriksson et al., 1985; Eriksson and Wistrand, 1986). Also Cl^- secretion is rapidly blocked by K^+-free (Marshall and Bryson, 1998) and Na^+-free salines (Marshall, 1981), consistent with NKCC mediation of Cl^- transport. Finally, NKCC1 expression (by qRT-PCR) and protein abundance (by western analysis) are increased sequentially after transfer of killifish from brackish water to seawater but not to freshwater (Scott et al., 2004), supporting NKCC1 as part of NaCl secretion in seawater.

NKCC is well known to be regulated by phosphorylation (reviewed by Flatman, 2002) and is activated by cell shrinkage in many cell types (Hoffmann and Dunham, 1995). In the inhibition of NaCl secretion by hypotonic shock, it is unlikely that transmural salt transport inhibition would act by blockade of solute exit at the apical membrane, although the CFTR homologue is an anion channel that is phosphorylated by protein kinase A (Marshall et al., 1995; Singer et al., 1998), because this would exacerbate the cell swelling (whereas inhibition of NKCC would actually aid in regulatory volume decrease). NKCC1 is found to be inactivated after cell swelling in various cells, such as Ehrlich ascites tumour cells (Krarup et al., 1998), thus hypotonicity would dephosphorylate NKCC and decrease NaCl entry, to reduce cell swelling and inhibit transepithelial Cl^- secretion. Presumably hypotonic shock also initiates a regulatory volume decrease involving activation of K^+ and Cl^- exit *via* independent channels, as in Erhlich ascites tumour cells (Hoffmann, 2000) or KCl cotransport, as in erythrocytes (Ellory et al., 1998). Conversely, hypertonic shock would shrink cells, phosphorylate NKCC, evoke regulatory volume increase and increase transepithelial Cl^- secretion. The mechanisms for NKCC phosphorylation/dephosphorylation are unknown, although several kinases have been found to be involved in various cell types (see Hoffmann and Dunham, 1995). In the killifish operculum myosin light chain kinase (MLCK) and protein kinase C (PKC) were shown to be involved in the activation of NKCC1 (Hoffmann et al., 2002) but the PKC isoform involved was not determined.

Prostaglandins are known also to be involved in cellular osmotic responses. In the vertebrate cell line Ehrlich ascites tumour cells, it has been demonstrated that cytoplasmic phospholipase A_2 ($cPLA_2$) is activated by hypotonic shock, resulting in increase of arachidonic acid release and increased eicosanoid synthesis (Hoffmann, 2000). The eicosanoid prostaglandin E_2 (PGE_2) is known to rapidly inhibit Cl^- secretion in the opercular membrane of *Fundulus heteroclitus* (Eriksson et al., 1985; Van Praag et al., 1987; Evans et al., 2003) and the tissue metabolizes several different eicosanoids, including prostaglandins, leukotrienes and hydroxyeicosatetraenoic acids (Van Praag et al., 1987). While thromboxanes and carbaprostacyclin (a PGI2 analogue) do not affect ion secretion rate, there appear to be both inhibitory and stimulatory PGE2 receptors in the killifish opercular epithelium (Evans et al., 2004). Cycloxygenase is immunolocalized not to the epithelial cells, but to the vascular space in the gill filaments (Evans, 2002), a localization more in tune with a vasoactive response, as has been suggested by Sundin and Nilsson (2002). The prostaglandin effect may be downstream of endothelin and nitric oxide agonists affecting cycloxygenase-2 (Evans et al., 2003). Therefore the opercular

epithelium was examined to see if hypotonic shock was affected by prostaglandins.

Because p38 MAPK and JNK have been implicated in volume responses of human leukemia cells (Pandey et al., 1999), Ehrlich ascites tumour cells (Pedersen et al., 2002) and rat hepatocytes (vom Dahl et al., 2001), we examined blockers of p38 MAPK and sought to identify this kinase using antibodies directed to human phosphorylated p38 MAPK and phosphorylated JNK. In *Fundulus heteroclitus* gill cells from animals exposed to osmotic shock, Kültz and Avila (2001) observed changes in activity and abundance of stress-associated protein kinase 1 (SAPK1, also known as Jun N-terminal kinase, JNK) and SAPK2 (p38 MAPK). In addition, a recently identified kinase that co-immunoprecipitates with NKCC1, a stress associated, Ste20/sps1-related proline-alanine-rich protein kinase SPAK (also known as PASK; Piechotta et al., 2002) is a prime candidate for involvement in NKCC1 regulation. We investigated expression of SPAK using an antibody directed against human SPAK. This heterologous approach is *via*ble in this case, because the degree of sequence similarity at the amino acid level between teleost (from genome libraries for Fugu, *Takifugu rubripes* and zebrafish, *Danio rerio*) and human protein kinases is greater than 90%, yet subclasses of protein kinases from one species have very low similarity, only 40–50%. As a result, we expected that the human directed antibodies should still detect the correct target enzymes in teleosts. Protein tyrosine kinase inhibition mimics the hypotonic response (Marshall et al., 2000) and it is well known that tyrosine kinases are involved in the RVD response in other cell types, e.g. Ehrlich ascites tumour cells (Hoffmann, 2000), thus we examined further the role of this enzyme in volume responses and extended this to include focal adhesion kinase (FAK), a ubiquitous scaffolding protein with a tyrosine phosphorylation site (Pandey et al., 1999). Hence the goal of the study was to begin uncovering the mechanisms of transport regulation, particularly of NKCC1, in chloride cells in response to osmotic stress using a combination of pharmacology, immunoblot and immunocytochemistry.

Materials and methods

Animals

Adult killifish (*Fundulus heteroclitus* L.) of both genders were captured in Antigonish estuary (Nova Scotia, Canada), transferred to indoor holding facilities and adapted to brackish water (salinity 10 g l^{-1}) for at least 10 days at 20-25°C and ambient photoperiod under artificial light. Fish were then moved to sectioned aquaria for a further 7 days (each section containing a pair of fish). During the acclimation period, fish were fed marine fish food blend (Nutrafin flakes; R. C. Hagen, Montreal, Canada) twice daily at a rate of 1.0 g 100 g^{-1} body mass day^{-1}.

Bathing solutions

Both opercular epithelia were dissected for electrophysiology of ion transport (below) and placed in a modified Cortland's saline (composition 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 and glucose 5.55, pH 7.8, when equilibrated with a 99% O_2/1% CO_2 gas mixture. Hypotonic shock was applied by flushing of the hemi-chambers with diluted Cortland saline at 244 mOsm kg^{-1}, a reduction of 60 mOsm kg^{-1} from the normal 304 mOsm kg^{-1}. Hypertonic shock, used in some cases, was applied by flow through of regular Cortland saline with 60 mmol l^{-1} mannitol added to increase osmolality to 364 mOsm kg^{-1}. The solution changes were performed symmetrically to avoid possible effects of asymmetrical solutions.

Electrophysiology

The opercular epithelium was removed and mounted in a modified Ussing chamber as described previously (Marshall et al., 1998) except that the epithelium was dissected without the nerve supply. The epithelium was supported by a nylon mesh and pinned out over the circular aperture (0.125 cm^2) with the rim area lightly greased and bevelled to minimize edge damage. In the membrane chambers, the following epithelial electrophysiological variables were monitored: transepithelial potential V_t (mV), transepithelial resistance R_t (Ω cm^2) and short-circuit current I_{sc} $\mu A cm^{-2}$). I_{sc} is expressed as positive for secretion of anions and is equivalent to the net secretion of Cl^- (Degnan et al., 1977). Epithelia were left at open circuit and were clamped to 0 mV for short periods to allow recording of I_{sc}. A current-voltage clamp (D. Lee Co., Sunnyvale, CA. USA or WP Instruments Sarasota, FL, USA; DVC 1000) was used to measure the epithelial variables. A control period of 1 h established the resting I_{sc}, after which drug additions and hypotonic shock tests were performed.

Pharmaceuticals

The protein kinase A inhibitor N-2-P-bromocinnamyl-aminoethyl-5-isoquinolinesulfoniamide (H-89), the protein kinase C inhibitor that is selective for subtypes α and β (Way et al., 2000) Gö6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole] and the protein kinase C inhibitor chelerythrine chloride were obtained from LC Laboratories, Woburn MA, USA and were dissolved in water (H-89 only) or a minimal volume of dimethylsulfoxide (DMSO; solvent for Gö6976 and chelerythrine). The β-adrenergic agonist isoproterenol (10 μmol l^{-1} in saline, serosal side) was used to test the efficacy of the PKA inhibitor H-89. H-89 (1.0 μmol l^{-1}) was added to the basal side, incubated for 30 min, then 10 μmol l^{-1} isoproterenol was added; parallel control membranes received no H-89. The p38 MAPK inhibitor SB203580, the protein phosphatase inhibitor okadaic acid and a second batch of Gö6976 were obtained from Calbiochem (San Diego, USA) and were all dissolved in a minimal volume of DMSO. Maximum amount of DMSO added with drug was 0.75% of chamber volume, a level without noticeable effect on the control membranes. Each drug was added to the serosal side of the opercular membrane, while the drug vehicle was added

to a parallel running paired control membrane from the same animal.

Preparation of extracts

Three pairs of opercular- and gill epithelia, from three different animals, were used in preparation of each sample. Dissected opercular and gill epithelia were transferred to boiling sodium dodecyl sulphate (SDS) lysis buffer (10 mmol l^{-1} Tris-HCl pH 7.4, 1% SDS) and minced, using scissors. The minced tissue was homogenized using a homogenizer, then boiled, frozen at –80°C, thawed and sonicated. The extracts were centrifuged for 5 min at 12,000 ***g*** and 5°C. Protein concentration was determined spectrophotometrically using the detergent-compatible Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Proteins were isolated from the crude extract by trichloroacetic acid (TCA) precipitation. TCA were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). To 1.0 ml samples containing equal amounts of protein were added 200 µl of ice cold 100% TCA solution and the samples incubated on ice for 30 min. Precipitated protein was collected by centrifugation for 10 min at 12 000 ***g***. The supernatant was discarded and the pellet washed three times in 500 µl ice-cold acetone, then air dried. The dry pellet was resuspended in NuPAGE 4 × lauryl dodecyl sulphate (LDS) sample buffer (Invitrogen, Carlsbad, CA, USA) supplemented with dithiothreitol (DTT) to 0.05 M final concentration.

SDS-PAGE and western blotting

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; precast NuPAGE 10% Bis-Tris gels and all NuPAGE products were purchased from Invitrogen) and electro-transferred to nitrocellulose membranes (Invitrogen), using NuPAGE transfer buffer. After transfer, the nitrocellulose membranes were incubated in blocking buffer (pH 7.5 Tris-buffered saline plus 0.1% Tween 20 (TBS-T), supplemented with 5% non-fat dry milk) for 1 h at room temperature or overnight at 4°C. The primary, polyclonal antibodies against p38 MAPK, phospho-p38 MAPK and phospho-JNK were purchased from Cell Signalling Technology Inc. (Beverly, MA, USA). The SPAK and OSR1 antibodies were a kind gift from Dr E. Delpire (Piechotta et al., 2002). All antibodies were diluted 1:100 and applied overnight at 4°C. The membrane was then washed four times in TBS-T for 5–15 min each. The goat anti-rabbit, alkaline phosphatase-conjugated secondary antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) was diluted 1:500 in blocking buffer and applied for 1 h at room temperature. The membranes were then washed as described above. Immunoreactive bands were detected using 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) membrane phosphatase substrate (KPL, Gaithersburg, MD, USA). A HP Scanjet 4600 (Hewlett Packard, Palo Alto, CA, USA) and the UN-SCAN-IT gel version 5.1 for Windows (Silk Scientific Corp., Salt Lake City, Utah, USA) software were used for quantification of immunoreactive bands and estimation of molecular masses.

Immunocytochemistry

The primary antibody for detection of Na^+,K^+,$2Cl^-$ cotransporter (NKCC) was T4 (Lytle et al., 1992; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), an antibody to the carboxyl region of NKCC that has been shown to bind to several isoforms of NKCC across several species (Haas and Forbush, 1998; Wilson et al., 2000) including killifish opercular membrane (Marshall et al., 2002). The secondary antibodies were goat polyclonal anti-mouse IgG conjugated to an Oregon Green 488 fluorophore and the same antibody conjugated to one of Alexa Fluor 546 or Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA). Primary antibody against SPAK was a rabbit polyclonal anti-mouse SPAK (residues 424-556; a gift of E. Delpire) (Piechotta et al., 2002). Primary antibody against phosphorylated FAK was rabbit polyclonal anti-hFAK[pY^{407}] (Biosource Int. Camarillo CA, USA) where the highly conserved epitope region is known to be 100% similar among human, mouse, rat, chicken and *Takifugu*. Secondary antibodies were goat anti-rabbit polyclonal conjugated with Oregon Green 488 and the same antibody conjugated with either Alexa Fluor 546 or 594 (Molecular probes, Eugene OR, USA).

Opercular epithelia were dissected without the dermal chromatophore layer and pinned to modeller's wax. For genistein pretreatment before pFAK antibody detection, paired membranes were incubated in aerated Cortland's saline for 2 h with 0.14% v/v DMSO vehicle or 100 mmol l^{-1} genistein in an equivalent volume of DMSO. Preparations were rinsed three times in rinsing buffer comprising 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 in PBS (TPBS), where PBS is phosphate-buffered saline, composition in mmol l^{-1}: NaCl 137, KCl 2.7, Na_2HPO_4 4.3, KH_2PO_4 1.4, pH 7.4. The membranes were fixed for 3 h at –20°C in a formaldehyde-free 80% methanol/20% dimethyl sulfoxide (DMSO) fixative. The methanol was used as a dehydrating agent and the DMSO as a cryoprotective agent. The membranes were rinsed three times, then immersed in a blocking solution with 5% normal goat serum (NGS)/0.1% BSA/0.05% TPBS, pH 7.4 for 30 min at room temperature in the dark and incubated in the primary antibody (8 µg ml^{-1} in 0.5% BSA in PBS) overnight at 4°C; parallel control tissues received no primary antibody. Control and test membranes were then rinsed three times and exposed to the secondary antibody (1:50 in 0.5% BSA in PBS), singly and in combination for 5 h at 4°C. After three final rinses the membranes were mounted in mounting medium (Geltol, Immunon Thermo Shandon, Pittsburgh, PA, USA). Mitochondria-rich cells were identified, using bright-field DIC microscopy, as large spheroidal cells with finely granular cytoplasm and centrally placed nucleus, confirmed by mitochondrial dye (Mitotracker, Molecular Probes, Eugene OR, USA). Slides were viewed in single blind fashion and

images collected with a laser confocal microscope (Olympus, Markham, ON, Canada; model FV300). In each opercular membrane, randomly selected Z-stack series were collected at 40×, zoom of 3.0 and with optical sections of 1.0±0.05 μm.

Statistical analyses

Data are expressed as the mean ± 1 S.E.M. Statistical significance was determined by paired *t*-tests between test and control membranes with significance ascribed if $P<0.05$, in a two-tailed test. For the immunoblot results, a one sample *t*-test, two-tailed, was applied comparing fold-activation relative to a reference and normalized for nonspecific kinase activation using controls incubated for the same time interval.

Results

Hypotonic shock

The initial response to hypotonic shock is a rapid massive reduction in I_{sc} that remained inhibited in steady state for at least 2 h. Typically we used a 1 h exposure (Fig. 1A). This I_{sc} inhibition could be the result of shut down of apical CFTR channels, basolateral NKCC, or the basolateral Na pump. However, shutdown of CFTR channels without changes to the other transporters would exacerbate the cell swelling, and inhibition of the Na pump would degrade the intracellular K levels and jeopardise the cell, so these actions are unlikely. More likely is a rapid inhibition of NKCC cotransporters that carry solutes into the cell. Inhibition of NKCC after hypotonic shock would simultaneously inhibit transmural Cl^- secretion and moderate the cell swelling. If, after a short 5 min interval of hypotonic shock, the isosmotic bathing solution was restored, no overshoot in I_{sc} occurred (Fig. 1B). However, longer exposures to hyposmotic stress yielded an increasing overshoot in I_{sc} when the isotonic solutions were restored (e.g. Fig. 1A) that reached 40 μA cm^{-2} at 120 min of exposure to hypotonic conditions (Fig. 1B). The overshoot indicates that the cells have responded to the shock by adjustment of the cellular compartment, probably through a regulatory volume decrease. This protocol formed the basis of pharmacological testing, where the drug was added to a test membrane while the paired control membrane from the same animal served as the parallel running control for a 1 h hypotonic shock followed by return to isosmotic conditions for a 1 h recovery. The degree of inhibition, magnitude and timing of the overshoot in current as well as transepithelial voltage and resistance were monitored.

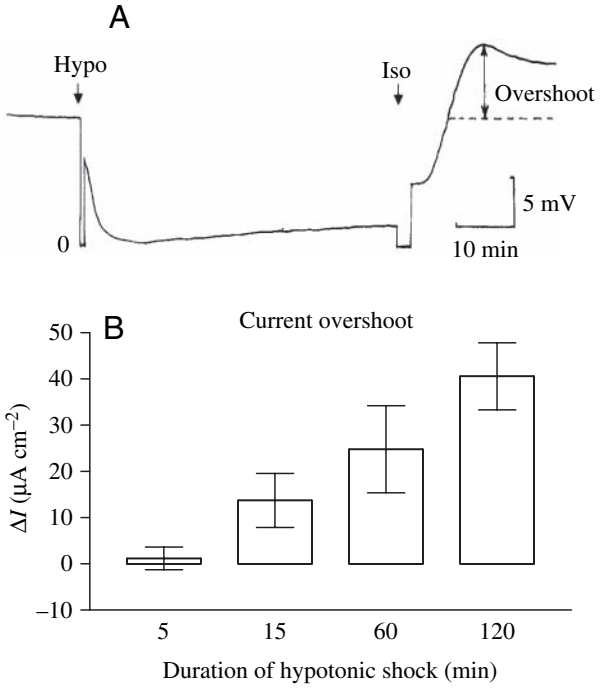

Fig. 1. Effect of hypotonic shock in isolated opercular epithelia mounted in symmetrical saline. (A) Example trace of time course of hypotonic shock on V_t, a 1 h duration period in hypotonic conditions and overshoot after restoration of isotonic conditions. (B) The size of the overshoot is a function of the period of hypotonic exposure; short periods yield no overshoot, longer periods yield larger overshoot in current, indicative of regulatory volume decrease during long hypotonic exposure.

Prostaglandin and PKA

We tested whether the cycloxygenase inhibitor indomethacin could prevent the initial current decrease in I_{sc} and the secondary increase in resistance after hypotonic stress. However, indomethacin had no detectable impact on the initial decrease in I_{sc} or resistance increase (Fig. 2), thus involvement of prostaglandins in the volume response is unlikely.

The PKA inhibitor (H89, 1.0 μmol l^{-1}) significantly reduced the augmentation of chloride secretion produced by 10.0 μmol l^{-1} isoproterenol in opercular membranes from 130±22 μA cm^{-2} to 21±3.3 μA cm^{-2} ($P<0.001$, $N=5$), thus confirming the efficacy of this kinase inhibitor in teleost systems. However, previous cAMP assays (Marshall et al., 2000) showed no change in cAMP levels with hypotonic shock, thus PKA appears not to be involved in the volume response.

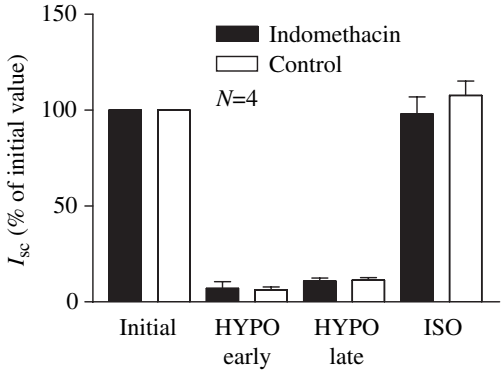

Fig. 2. The cycloxygenase inhibitor indomethacin (0.1 mmol l^{-1}) had no detectable effect on the hypotonic response (HYPO) and no effect on the current rebound when isotonic conditions were restored (ISO).

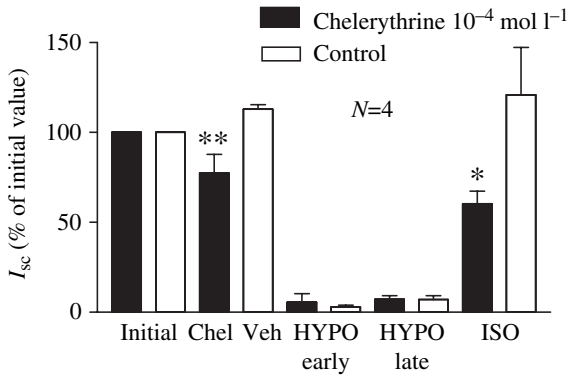

Fig. 3. The protein kinase C inhibitor chelerythrine (Chel) significantly inhibited membrane current compared to vehicle (Veh) treatment (**P<0.01, paired t-test) and significantly reduced the recovery of current after isotonic (ISO) solutions were restored (*P<0.05, paired t-test).

Protein kinase C

The general PKC inhibitor chelerythrine significantly inhibited the resting membrane current but had little effect on the hypotonic shock response (Fig. 3). Chelerythrine greatly reduced the recovery of the membrane current when isotonic solutions were restored (Fig. 3). There was no significant change in transepithelial resistance with chelerythrine (data not shown). To help determine the isoform of PKC that might be involved, we used a more specific PKC inhibitor, Gö6976, which selectively inhibits the calcium-dependent conventional PKC (cPKC) isoforms (α and β1) (Way et al., 2000). However, Gö6976 at 1.0 and 10 μmol l^{-1} did not reduce the resting I_{sc} and it did not block the hypotonic response (current reduction in vehicle was –81.6±19.6 *versus* –100.3±30.7 μA cm^{-2}, N=3 animals on three experiment days) and it did not block the recovery afterwards when isotonic solutions were introduced. There was no significant change in transepithelial resistance with Gö6976 (data not shown).

p38 mitogen activated protein kinase

The p38 MAPK inhibitor SB 203580 is considered to be highly specific (Davies et al., 2000; Bain et al., 2003) and we have used this inhibitor to investigate whether this MAPK is involved in hypotonic inhibition and hypertonic activation of the Cl^- current. Whereas SB203580 had no effect on resting current (Fig. 4), the decrease in current was significantly larger in the SB203580-treated membranes (P<0.05). There was a decrease in the current overshoot when isotonic solutions were replaced (P<0.05) and a trend toward a smaller increase in current when hypertonic conditions were invoked. Transepithelial resistance increases with hypotonic shock and, with SB203580, the increase in resistance is larger (P<0.05), reflecting the larger decrease in current. This general pattern holds, so resistance results are not shown for the other treatments.

Activation of p38 MAPK was measured by western blot analysis in triplicate in tissues that were dissected and placed in hypotonic or isotonic bathing media for different times and scanned with reference to tissues incubated in isotonic conditions for the same time (Fig. 5). The antibody to p38 MAPK and that to the phosphorylated form of p38 MAPK detected a single band in the expected molecular mass range, 38–40 kDa. The p38 MAPK phosphorylation was significantly higher than controls at 5 min incubation in hypotonic media (eightfold, P<0.05, paired t-test compared to isotonic control level at each time), threefold at 30 min incubation in hypertonic media and eightfold by both hypertonic and hypotonic shock in a second phase response at 1 h (P<0.05) (Fig. 5A,B). Equal amounts of p38 MAPK were expressed in each sample of opercular epithelium, as measured with antibody to unphosphorylated p38 MAPK (Fig. 5A).

Gill and opercular epithelium tissue from long-term acclimated freshwater and seawater killifish yielded detectable expression of p38 MAPK western immunoblots for both epithelia and in both salinities (Fig. 6). There was significantly lower expression in the

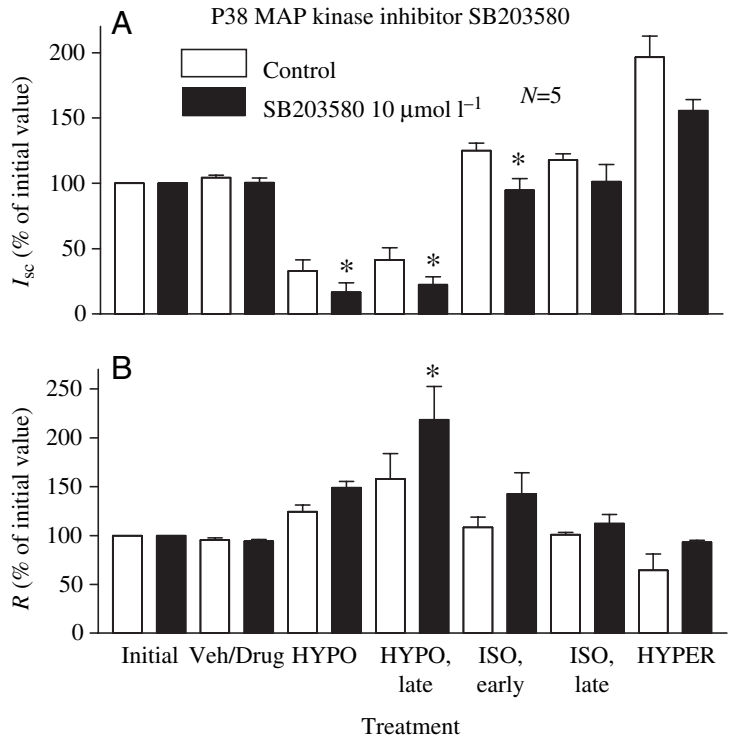

Fig. 4. The p38 MAPK inhibitor SB203580 had no effect on current (A) compared to the vehicle-treated membranes (Drug/Veh), but SB203580 significantly enhanced the inhibition of current by hypotonic shock (HYPO) and significantly reduced the recovery of current after restoration of isotonic conditions (ISO; *P<0.05, paired t-test). The transepithelial resistance (B) change mirrors the current changes, with SB203580 increasing the resistance rise compared to vehicle (*P<0.05, paired t-test) seen with hypotonic shock.

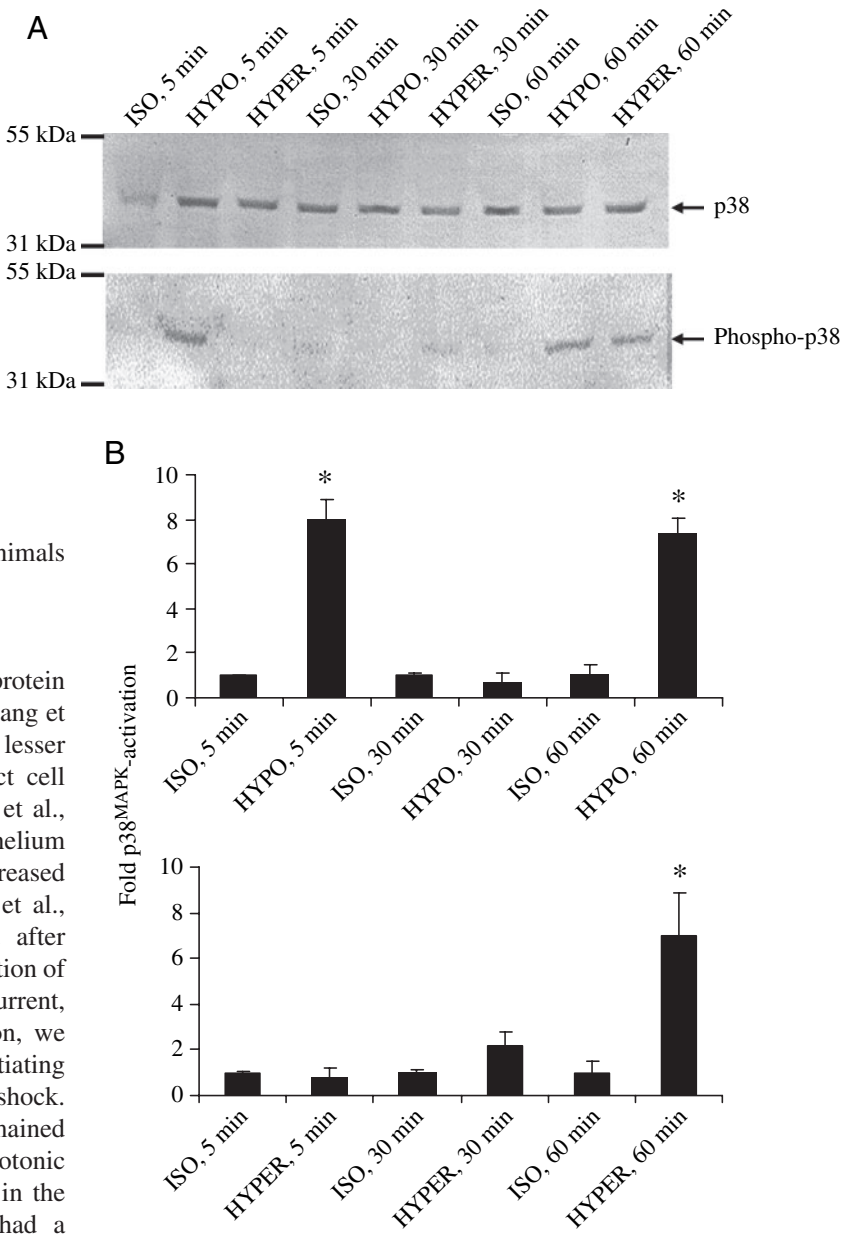

Fig. 5. Immunoblot of protein form opercular epithelia treated for different times with isotonic solution (control), hypotonic saline (Hypo) and hypertonic saline (Hyper). (A) Upper panel: p38 MAPK (38–40 kDa) was detected by antibody to any form of the protein. Lower panel: p38 MAPK detected by the antibody specific to the phosphorylated form of the protein. (B) Bar charts of quantitative scans averaged over three immunoblots where p38 MAPK phosphorylation was enhanced significantly (*P<0.05, paired t-test compared to isotonic control level at the respective time, N=3) by hypotonic shock at 5 min, by hypertonic shock at 30 min and by both hypertonic and hypotonic shock in a second phase response at 60 min.

gill and opercular membranes from seawater animals relative to tissue from freshwater acclimated fish.

Okadaic acid and protein phosphatase

Okadaic acid is an inhibitor of serine/threonine protein phosphatases (PP) that binds to the active site (Huang et al., 1997). It has highest affinity for PP2A and to a lesser extent for PP1 (Takai et al., 1993) and in intact cell systems also can act on PP4 and PP5 (Millward et al., 1999). It was previously shown in opercular epithelium that the PP1 and PP2A inhibitor calyculin A increased the steady state current significantly (Hoffmann et al., 2002) and potentiated the decrease in current after hypotonic shock (Zadunaiski et al., 1997). Application of okadaic acid significantly increased membrane current, compared to vehicle controls (Fig. 7). In addition, we found that okadaic acid had a marginal potentiating effect on the decrease in current after hypotonic shock. The current in control and test membranes remained inhibited for the hour. However, restoration of isotonic conditions did not restore the membrane current in the okadaic acid-treated membranes, but controls had a normal overshoot in current (Fig. 7).

Protein tyrosine kinase

The protein tyrosine kinase (PTK) inhibitor genistein, but not the inactive analogue daidzein (Marshall et al., 2000) inhibit chloride secretion in seawater killifish opercular epithelia (P<0.01, Fig. 8; Marshall et al., 2000). However, the effect is level dependent. If the membranes are first inhibited with the α-adrenergic agonist clonidine (1.0 μmol l^{-1}), genistein instead increases chloride secretion (P<0.05), as I_{sc} (Fig. 8). Thus the effect of genistein is to release the membrane from steady-state stimulated or inhibited states, allowing the current to move to an intermediate level.

Jun N-terminal kinase

Antibody to the phosphorylated form of JNK (pJNK) was used in western analysis to determine if JNK was activated by hyper and hypotonic shock (Fig. 9). With time, there appeared to be an increase in pJNK in the isotonic controls, probably an effect of repeated disturbance of the fish before tissue collection. Therefore, the time controls in isotonic media were ascribed a normalized 100% and the treated tissues (from animals sampled at the same time) were compared to this control. There was a modest activation of pJNK by hyper- and hypotonic shock at 5 min (P<0.05) and again at 30 min incubation in hypertonic media, compared to parallel running control tissues in isotonic conditions. The 63 kDa isoform detected here is the same protein described as SAPK1 by Kültz and Avila (2001).

The stress associated protein kinases SPAK and OSR1

Western analysis of OSR1 and SPAK in gill and opercular

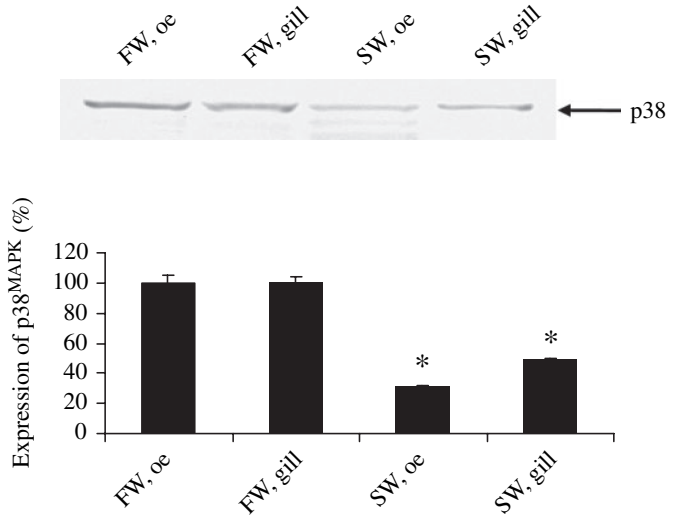

Fig. 6. The p38 MAPK is present in opercular epithelium and gill epithelial tissue in fully acclimated freshwater and seawater animals. Scans averaged over three immunoblots (top) indicate a lower level of expression of p38 MAPK (bottom) in seawater relative to freshwater levels in both gill and opercular epithelium (*P<0.05, t-test, N=3).

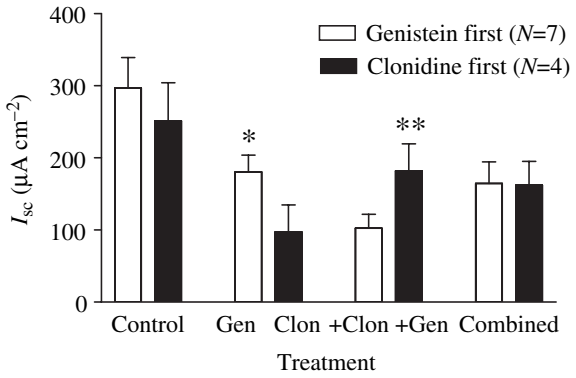

Fig. 8. The protein tyrosine kinase inhibitor genistein (Gen; 0.1 mmol l^{-1}) if added to membranes with high levels of current (salt water) caused a significant inhibition (**P<0.01, unpaired t-test) compared to vehicle controls. However, if the current was inhibited first by clonidine (Clon; α_2 adrenergic agonist, 1.0 μmol l^{-1}), genistein instead increased the current to an intermediate level (*P<0.05). The inactive analogue daidzein (0.1 mmol l^{-1}) has no inhibitory effect (Marshall et al., 2000).

membrane epithelial cells demonstrated that OSR1, the 66 kDa isoform and a truncated form, were present in freshwater and seawater gill and opercular epithelium (Fig. 10A), and that expression in freshwater-acclimated animals was significantly greater than in seawater-acclimated animals, by two- to fivefold for gill and opercular membrane, respectively. There were positive indications of SPAK expression in seawater and freshwater opercular epithelium and gill tissue (Fig. 10B) with modestly higher expression of SPAK in freshwater-acclimated animals. Immunocytochemistry demonstrated that NKCC is present in the mid to lower levels of mitochondria-rich cells in the opercular membrane, at the level of the nucleus and to the basal side of the cells (Fig. 11A). This distribution is similar to the distribution of SPAK immunofluorescence (Fig. 11B) and in most cases there is exact colocalization of NKCC and SPAK (yellow colour in Fig. 11C; line scan in Fig. 11D), consistent with juxtaposition of the two proteins (N=8 animals on 6 experiment days). In seawater opercular epithelia, there was also colocalization of NKCC and OSR1 (Fig. 11E–H) with all mitochondria-rich cells showing colocalized immunofluorescence of the two proteins (N=3 animals on 3 experiment days).

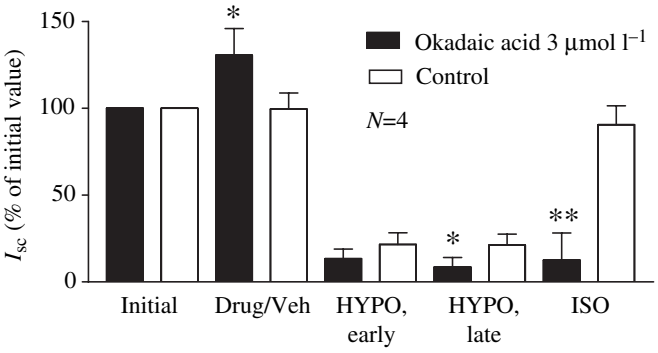

Fig. 7. The protein phosphatase inhibitor okadaic acid significantly increased membrane current initially (*P<0.05, paired t-test), had a marginal potentiating effect on the magnitude of the hypotonic inhibition of current (*P<0.05, paired t-test) and entirely blocked the recovery of current (**P<0.02, paired t-test) after restoration of isosmotic solutions.

Focal adhesion kinase

Immunocytochemistry using antibody for the phosphorylated form of the scaffolding protein focal adhesion kinase (pFAK) revealed in 11 experiments on 11 animals, positive staining for pFAK (green fluorescence in Fig. 12A) with the cotransporter NKCC (red fluorescence in B; four experiments on four animals) in mitochondria-rich cells of seawater killifish opercular membranes. Furthermore, FAK appeared in all mitochondria-rich cells and was highly colocalized with NKCC (four experiments on four animals) in all mitochondria-rich cells in the membrane (yellow fluorescence in Fig. 12C). Genistein pretreatment (100 μmol l^{-1}) of opercular epithelia before addition of the anti-pFAK antibody completely eliminated pFAK fluorescence (Fig. 12D) while the genistein control tissue that received 0.14% DMSO vehicle had positive pFAK fluorescence (not shown).

Discussion

Protein kinase A (PKA) and H-89

The present results confirm that H-89 blocks the cAMP/PKA stimulation evoked by isoproterenol (Hoffmann et al., 2002). H-89 however, does not block the hypertonic stimulation of

Cl^- secretion by the killifish opercular epithelium (Hoffmann et al., 2002) and hypotonic inhibition of Cl^- secretion is not accompanied by changes in tissue cAMP (Marshall et al., 2000), therefore other kinase-stimulated pathways are probably involved.

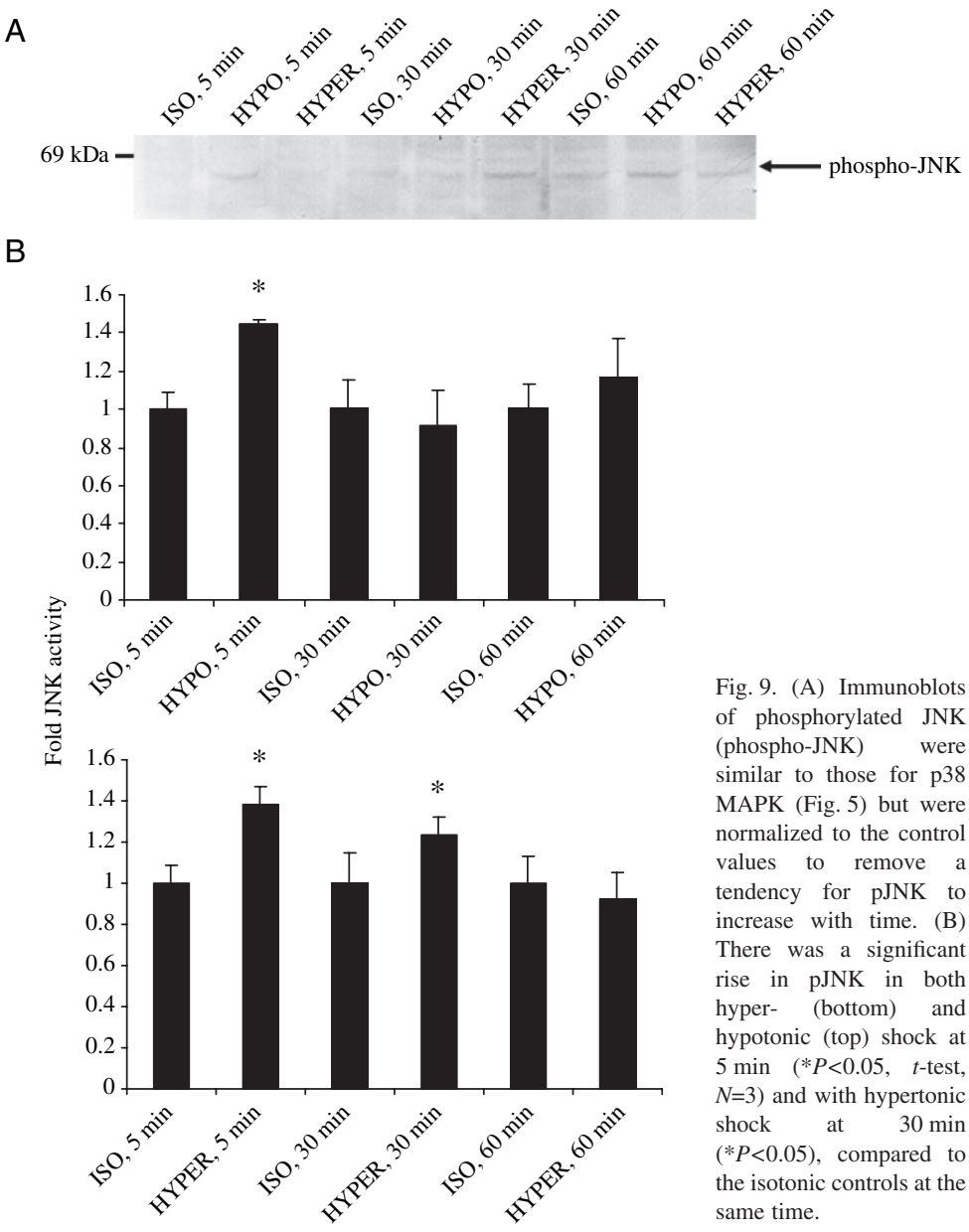

Fig. 9. (A) Immunoblots of phosphorylated JNK (phospho-JNK) were similar to those for p38 MAPK (Fig. 5) but were normalized to the control values to remove a tendency for pJNK to increase with time. (B) There was a significant rise in pJNK in both hyper- (bottom) and hypotonic (top) shock at 5 min (*P<0.05, t-test, N=3) and with hypertonic shock at 30 min (*P<0.05), compared to the isotonic controls at the same time.

p38 MAPK and JNK

Multiple protein kinases are activated following hypertonic stress in mammalian cells including the MAPK subfamilies Erk1, Erk2, p38 MAPK and JNK (Pandey et al., 1999; Roger et al., 1999; Duzgun et al., 2000; Pedersen et al., 2002). The p38 MAPK has been shown to be induced by hypertonic shock in corneal epithelial cells (Bilden et al., 2003). As well, p38 MAPK is involved in the regulatory volume increase process (Roger et al., 1999; Sheikh-Hamad et al., 1998).

In killifish, gill SAPK2 (=p38 MAPK) is enhanced by salinity transfer with a fivefold increase in the expression of SAPK2 1 h after transfer from freshwater to seawater (Kültz and Avila, 2001). Our data confirm the activation of SAPK2/p38 MAPK in teleost gill at 1 h and extend previous results by revealing an early, 5 min eightfold activation by hypotonic shock only. The differential activation of p38 MAPK with hypotonic shock at 5 min implicates this kinase in the swelling-induced inhibition of chloride secretion that is happening simultaneously. But the p38 kinase inhibitor SB203580 exacerbates the hypotonic response in the opercular membranes, suggesting that p38 kinase is still involved with activation of the Cl^- secretion. Consistent with this, the p38 inhibitor also blocks the overshoot after an hour when isotonic solutions are restored and p38 activation is again elevated.

Protein kinase C and myosin light chain kinase

Our results reported here showing inhibition of the hypertonic response by chelerythrine confirm our previous findings (Hoffmann et al., 2002) and support the idea of PKC involvement in volume responses. In eel intestine, chelerythrine and calyculin A were potent inhibitors of the activation of NKCC-mediated ion uptake in an RVI response to hypertonic shock (Lionetto et al., 2002). In human cervical cancer cells, hypotonic shock evokes activation of extracellular signal activated kinase (ERK1 and ERK2) and p38 MAPK through a PKC-dependent pathway that also involves intracellular calcium (Shen et al., 2001). In rabbit proximal tubule cells, PKC is translocated to the plasma membrane following hypotonic shock (Liu et al., 2003). Meanwhile, hyperosmolality activates some PKC isoforms in NIH/3T3 cells (Zhuang et al., 2000).

There has been some discussion about the specificity and efficacy of chelerythrine, that it is effective in vivo against cPKCα,β,γ but less so against nPKC isoforms (Keenan et al., 1997) and that it is ineffective in some in vitro systems (Lee et al., 1998). We therefore also tested a more selective PKC inhibitor, Gö6976, which specifically inhibits the calcium-

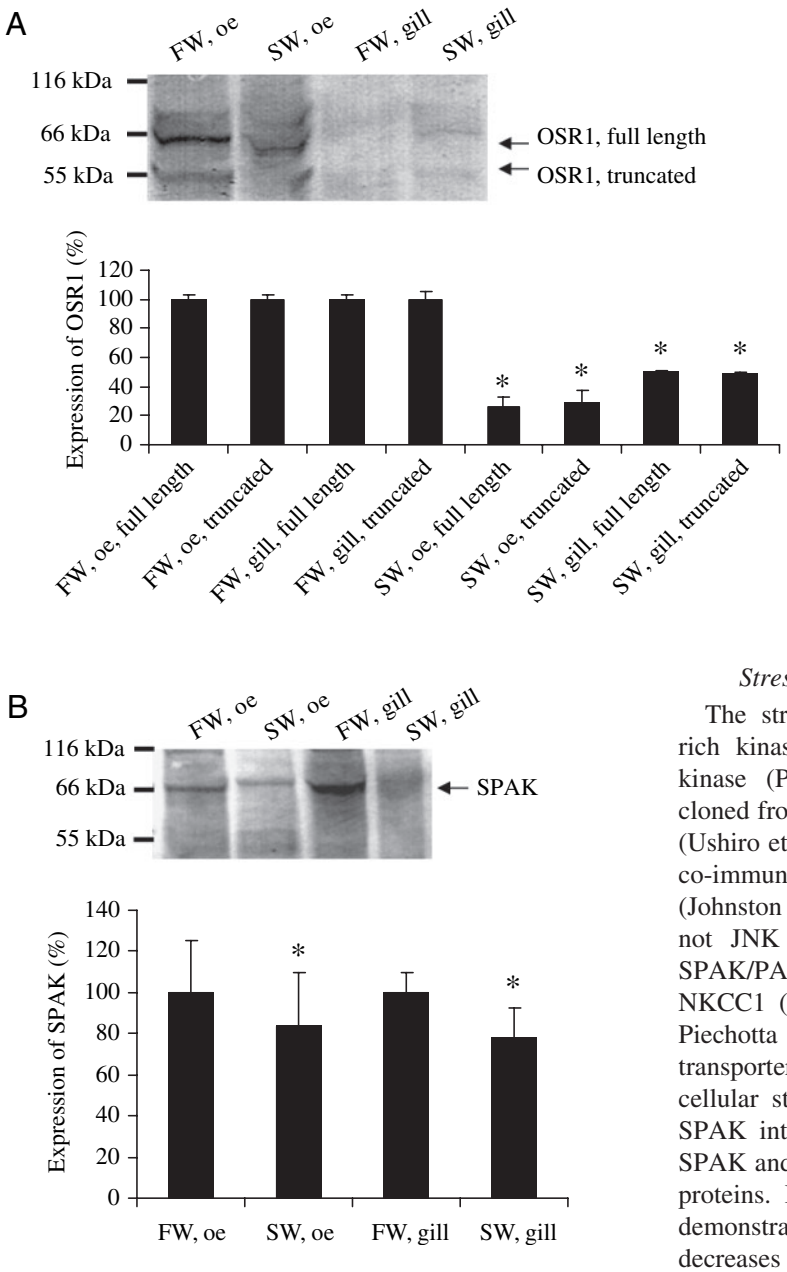

Fig. 10. Immunoblot of the stress-associated proteins OSR1 (A) and SPAK (B), comparing expression in freshwater (FW)- and seawater (SW)-acclimated animals. Quantitative western analysis revealed significantly higher level of OSR1 and SPAK expression in FW- as compared to SW-acclimated animals for the gill and opercular epithelium (oe; N=3, *P<0.05) with the difference in OSR1 expression being more marked than that for SPAK.

dependent conventional PKC (cPKC) isoforms (α and β1) (Way et al., 2000). Because Gö6976 inhibited neither the hypotonic response nor its recovery, it appears that the cPKC isoforms (α and β1) are not involved, rather that other isoforms of cPKC or members of the novel PKC (nPKC) family are involved. It has been shown in NIH/3T3 cells that hyperosmolarity activates both cPKC and nPKC families of kinases (Zhuang et al., 2000). Hypotonic stress in rabbit proximal tubule cells specifically stimulates translocation of certain isoforms of PKC to the plasma membrane, notably PKCα, PKCε and PKCζ (Liu et al., 2003). NKCC is activated by PKC in rabbit tracheal epithelium cells (Liedtke and Thomas, 1996) and the isoform is the PKCδ (Liedtke et al., 2003). While it is clear that PKC is involved in chloride cell volume responses, it is possible that PKCδ, PKCε and/or PKCζ are the forms involved with chloride cells, but this awaits positive identification of the PKC isoform(s). Of particular interest is PKCδ, which colocalizes with actin and NKCC1 in the periphery of airway epithelial cells. This complex could be represent the osmosensing mechanism (Liedtke et al., 2003).

Myosin light chain kinase (MLCK) inhibitors block the augmentation of Cl^- secretion by the opercular epithelium (Hoffmann et al., 2002)

Stress-associated protein kinases, SPAK and OSR1

The stress-associated Ste20/SPS1-related proline-alanine-rich kinase (SPAK) and proline-alanine-rich Ste20-related kinase (PASK) are homologous serine-threonine kinases cloned from human brain (Johnston et al., 2000) and rat brain (Ushiro et al., 1998), respectively. SPAK and PASK interact, co-immunoprecipitate and specifically activate p38 MAPK (Johnston et al., 2000; Piechotta et al., 2002) but apparently not JNK or ERK (Johnston et al., 2000). The kinases SPAK/PASK and OSR1 also co-immunoprecipitate with NKCC1 (Dowd and Forbush, 2002; Johnston et al., 2000; Piechotta et al., 2002), as well as other cation chloride co-transporters, such as KCC3 (Piechotta et al., 2002). These cellular stress proteins, together with the demonstration of SPAK interactions with p38 MAPK and NKCC1, point to SPAK and OSR1 functioning as stress responsive scaffolding proteins. In support of this notion, Piechotta et al. (2003) demonstrated that binding of SPAK to NKCC1 and p38 MAPK decreases during cellular stress. The amount of p38 MAPK coimmunoprecipitated with SPAK and the cotransporter significantly decreases upon cellular stress, while the binding of SPAK to NKCC1 remains unchanged.

In teleost fish there are similar genes. There are homologs for hOSR1 in zebrafish (*Danio rerio*) and puffer fish (*Takifugu rubripes*) genomes that are 81% and 77% identical at the amino acid level, respectively, and for hSPAK that are 74% and 73% identical, respectively. However, the nearest match to hSPAK in zebrafish is in fact the OSR1 homologue, so the zebrafish (freshwater stenohaline) may lack a SPAK homologue. It is also of interest that the marine puffer fish OSR1 homologue has a seven amino acid deletion in the centre of the catalytic region (VLMLTLQ at position 235-241), while the SPAK homologue is exactly homologous in the same region and there is a truncated (366 aa) SPAK homologue with 85% identity to the human SPAK that includes the seven amino acid sequence.

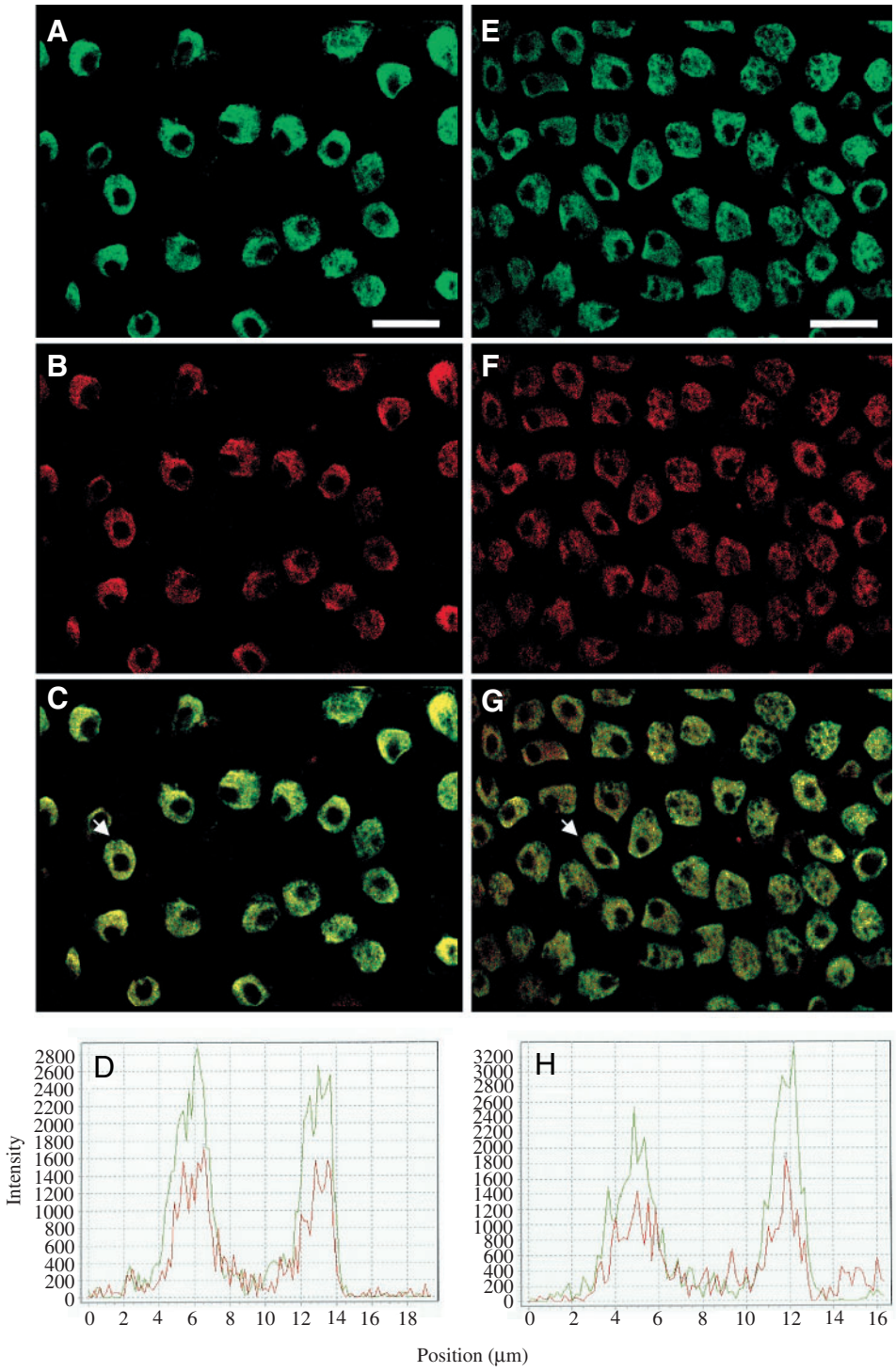

Fig. 11. Immunocytochemistry for NKCC (T4 mouse anti-hNKCC antibody with goat anti-mouse Oregon Green 488) and for SPAK (rabbit anti-SPAK polyclonal with goat anti-rabbit Alexa Fluor 594) visualized by confocal microscopy. (A) Anti-NKCC alone (green) (B) Anti-SPAK alone (red) (C) Same field as A and B but with both channels activated (red/green); exact colocalization is yellow. NKCC and SPAK appear in the same mitochondria-rich cells, not in the apical membrane but lower in the cell (here at the plane of the nuclei) and, in most areas are exactly colocalized (yellow in C). (D) A line scan of fluorescence intensity (arbitrary units) *versus* distance in μm across a cell (indicated by the arrow in C) shows good correspondence in the colocalization of SPAK (red line) and NKCC (green line) in the cytosol (peaks) and not in the nucleus (central region). (E–H) As A–D except that the primary antibody for the kinase was rabbit anti-OSR1 polyclonal, not SPAK. Bars, 20 μm.

This analysis suggests that SPAK may be more important in seawater osmoregulation.

In the mitochondria-rich chloride cell, SPAK and NKCC1 colocalize by immunocytochemistry and SPAK expression is detectable by immunoblotting in gill and opercular epithelial cells of seawater and freshwater acclimated animals, suggesting a role of the protein in NKCC1 regulation in salinity acclimation. There is significantly more SPAK expression in freshwater animals compared to seawater fish, consistent with the general trend for higher expression of kinases in freshwater (Kültz and Avila, 2001). Importantly, co-expression of wild-type SPAK/PASK with either human or shark NKCC1 increased co-transport activity in HEK cells, affecting phosphorylation of the regulatory residues T184/T189 in shark NKCC1 (Darman and Forbush, 2002; Dowd and Forbush, 2002), indicating a relationship between SPAK and NKCC1 activation in fish. Our results confirm this relationship and extend it to teleost chloride cells in both freshwater and seawater.

OSR1 is expressed in seawater opercular epithelium and gill, but at much lower levels than in freshwater-acclimated animals. Our OSR1 results are consistent with previous findings, where transfer of killifish to freshwater appears to upregulate expression of all MAP kinases, while transfer to hyperosmotic conditions does the reverse (Kültz and Avila, 2001). There was a clear colocalization of OSR1 with NKCC by immunocytochemistry, suggesting a strong role for OSR1 in seawater osmoregulation and its higher expression suggests also a role in freshwater osmoregulation. The lack of a SPAK homologue in zebrafish (above) also implicates OSR1 as being more important in stenohaline freshwater teleosts.

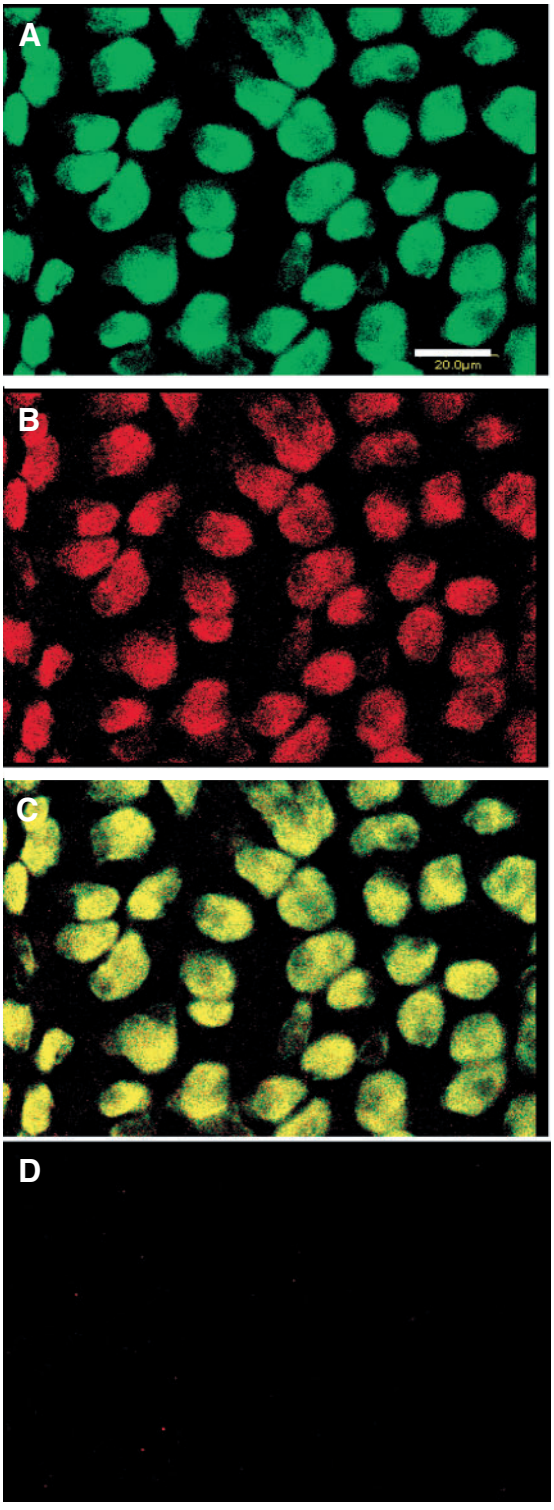

Fig. 12. Immunocytochemistry for phosphorylated focal adhesion kinase (pFAK) (primary: rabbit anti-phosphorylated human FAK; secondary: goat anti-rabbit Oregon Green 488) and for NKCC (primary: T4 mouse anti-hNKCC; secondary: goat anti-mouse Alexa Fluor 594). (A) pFAK immunofluorescence was present in all mitochondria-rich cells (green). (B) NKCC immunofluorescence (red) had a similar distribution to pFAK. (C) There was a high degree of colocalization of NKCC and phosphorylated pFAK (yellow). (D) Genistein pretreatment (100 µmol l^{-1}, for 1 h) to inhibit tyrosine kinase before addition of the primary anti-pFAK antibody eliminated pFAK immunofluorescence. Bar, 20 µm.

Protein phosphatase

While it is clear there is serine/threonine protein phosphatase (PP) involvement in the recovery from hypotonic shock, the subtype could be PP2a, PP1 or possibly PP4 and PP5, because the concentration of Okadaic acid used here on whole tissue was high enough to potentially affect all these phosphatase subtypes (Millward et al., 1999).

The cotransporter in shark rectal gland epithelium, a salt secreting epithelium that shares many functional aspects with teleostean salt-secreting cells of the gill and opercular membrane, has a protein phosphatase 1 (PP1) binding site in the amino terminus at residues 107-112, and PP1 co-immunoprecipitates with NKCC1 (Darman et al., 2001). Thus it is probable that PP1 may also be involved in dephosphorylation of NKCC1 in teleosts. In support of this notion, okadaic acid that blocks PP1 and related protein phosphatases, causes an increase in membrane current in the opercular epithelium. The effect of okadaic acid in blocking recovery from hypotonic shock requires an alternative explanation. The lack of recovery after blockade of protein phosphatases implies that there is a protein phosphatase whose activity is needed to unlock or release the regulatory complex from steady state inhibition.

Protein tyrosine kinase

Tyrosine kinases are activated during RVD in several different cell systems and a PTK inhibitor genistein inhibits RVD (for a review, see Hoffmann, 2000) and tyrosine kinases appears to play a role in the volume sensing mechanism. Genistein alters chloride secretion by opercular membranes in a level-dependent fashion, increasing chloride secretion of inhibited tissues (inhibited by α adrenergic agonist) and decreasing secretion in salt water stimulated tissues. Genistein appears to unlock the transport from steady state 'on' or 'off' conditions, yielding a moderate or intermediate level. Daidzein, the analogue of genistein that is not active on kinases, is without effect on the opercular membrane (Marshall et al., 2001). In a related system, the shark rectal gland (that normally secretes Cl^- at a low rate), genistein has been shown

to augment chloride secretion without activating cAMP (Lehrich and Forrest, 1995). Genistein instead evokes trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) into the apical membrane (Lehrich et al., 1998), but the mechanism is not clear. CFTR has numerous Ser/Thr phosphorylation sites as well as a tyrosine site that might be involved (Dahan et al., 2001). NKCC isoforms have two threonine residues that are phosphorylation sites and phosphorylation of these sites, detected by antibody directed against the phosphorylated form, occurs when NKCC is activated by cAMP *via* PKA (Flemmer et al., 2002). However, because there is no tyrosine phosphorylation site on NKCC, any action of tyrosine kinase must be indirect.

Focal adhesion kinase

The focal adhesion kinases are involved in membrane protein regulation and especially in cellular motility. FAK signalling is connected to its ability to become phosphorylated in response to integrin-mediated adhesion to Tyr-397. Integrin-FAK interaction permits further interactions with a number of different signalling effectors containing Src homology 2 (SH2) domains (Hanks et al., 2003; Gelman, 2003). FAK Tyr-397 is an autophosphorylation site that, when phosphorylated, defines an interaction surface for SH2 domains. Proteins found to interact with Tyr-397 include Src, Grb7, Shc, PLCγ and the p85 subunit of phosphoinositide 3-kinase. When Src is recruited to this site, additional tyrosine residues are phosphorylated. FAK associates with integrins *via* its N-terminal FERM domain and to talin and paxillin *via* the C-terminal focal adhesion targeting (FAT) domain (Gelman, 2003; Hauck et al., 2002). In hepatocytes, hypotonic shock activates a number of kinases including p38 MAPK, as in the opercular membrane, but does not affect phosphorylation of FAK (vom Dahl et al., 2003). Conversely, in fibroblasts, stretch-induced phosphorylation of FAK at Tyr-397 is critical to activation of p38 MAPK in response to cyclic stretch (Wang et al., 2001). Our results indicate FAK and NKCC colocalization is very close and correspondent in every mitochondria-rich cell in the tissue. Furthermore, genistein, which decreases Cl^- secretion in membranes with high current and increases I_{sc} in previously inhibited membranes, results in FAK dephosphorylation (Fig. 12). Our results suggest dephosphorylation of FAK on hypotonic shock connected to the rapid dephosphorylation and deactivation of NKCC. We speculate that FAK phosphorylation, presumably at Tyr-397, locks NKCC in steady state high or low activity levels and that FAK dephosphorylation is permissive to rapid changes in NKCC phosphorylation and activity level.

We observed rapid activation through phosphorylation of JNK in response to hypotonic shock and this observation is consistent with previous results of interactions between FAK and JNK. The FAK7/p130^{Cas} complex can mediate anchorage-dependent activation of JNK (Almeida et al., 2000; Oktay et al., 1999). Also, FAK and JNK co-localization was observed (Almeida et al., 2000). Igishi et al. (1999) suggest that activation of JNK by FAK is independent of the kinase activity of FAK. Rather, FAK-mediated recruitment of paxillin to the plasma membrane is sufficient for JNK activation.

A tentative model

Whereas there is no tyrosine phosphorylation site on NKCC1, FAK has a tyrosine phosphorylation site and is known to operate as a scaffolding protein, associated with membrane proteins and the cytoskeleton. The almost exact colocalization of NKCC with phosphorylated FAK demonstrated here supports the notion of FAK being a scaffolding protein in NKCC regulation in teleost mitochondria-rich cells. For instance colocalization of FAK with actin has implicated FAK in normal spreading of cultured intestinal epithelial cells (Ray et al., 2001). Thus FAK could be part of the regulatory complex with NKCC1, OSR1 and SPAK. Dephosphorylation of a tyrosine apparently 'unlocks' the complex, allowing NKCC1 to be phosphorylated and turned on (consistent with hypertonic shock and RVI) or, if the transport is already activated, allowing NKCC1 to be dephosphorylated and turned off (consistent with hypotonic shock and RVD). We hypothesize then, that phosphorylated FAK (phosphorylated on Tyr-397) somehow occludes phosphorylation sites on NKCC and locks the cotransporter in its present phosphorylation state. When FAK is dephosphorylated, the complex enters a dynamic state where phosphorylation or dephosphorylation is allowed. The activation of NKCC1 (that contributes to RVI) probably involves p38 MAPK, MLCK, PKC, OSR1 and SPAK terminating with phosphorylation of the two Ser/Thr sites on NKCC1. Conversely, the cascade that turns off NKCC1 (and contributes to RVD) probably involves JNK because it is activated by hypotonicity and a terminal protein phosphatase, possibly PP2A, as a means to dephosphorylate and deactivate NKCC1.

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