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CFTR Cl^- channel functional regulation by phosphorylation of focal adhesion kinase at tyrosine 407 in osmosensitive ion transporting mitochondria rich cells of euryhaline killifish

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SUMMARY

Cystic fibrosis transmembrane conductance regulator (CFTR) anion channels are the regulated exit pathway in Cl^- secretion by teleost mitochondria rich salt secreting (MR) cells of the gill and opercular epithelia of euryhaline teleosts. By confocal light immunocytochemistry, immunogold transmission electron microscopy (TEM), and co-immunoprecipitation, using regular and phospho-antibodies directed against conserved sites, we found that killifish CFTR (kfCFTR) and the tyrosine kinase focal adhesion kinase (FAK) phosphorylated at Y407 (FAK pY407) are colocalized in the apical membrane and in subjacent membrane vesicles of MR cells. We showed previously that basolateral FAK pY407, unlike other FAK phosphorylation sites, is osmosensitive and dephosphorylates during hypotonic shock of epithelial cells (Marshall et al., 2008). In the present study, we found that hypotonic shock and the α_2-adrenergic agonist clonidine (neither of which affects cAMP levels) rapidly and reversibly inhibit Cl^- secretion by isolated opercular membranes, simultaneous with dephosphorylation of FAK pY407, located in the apical membrane. FAK pY407 is rephosphorylated and Cl^- secretion rapidly restored by hypertonic shock as well as by forskolin and isoproterenol, which operate *via* cAMP and protein kinase A. We conclude that hormone mediated, cAMP dependent and osmotically mediated, cAMP independent pathways converge on a mechanism to activate CFTR and Cl^- secretion, possibly through tyrosine phosphorylation of CFTR by FAK.

Key words: cystic fibrosis transmembrane conductance regulator (CFTR), tyrosine phosphorylation, epithelial transport, teleost osmoregulation, metabolon, ion channel regulation, hypotonic, focal adhesion kinase (FAK).

INTRODUCTION

Cystic fibrosis is a genetic disorder of the channel and regulatory protein cystic fibrosis transmembrane conductance regulator (CFTR). An important characteristic of mammalian (Aleksandrov et al., 2007) and killifish (Singer et al., 1998) CFTR is its activation *via* cyclic AMP (cAMP) and protein kinase A (PKA), a pathway that terminates with serine and threonine residue phosphorylation in the regulatory (R) domain of CFTR protein, exon 13, nominally amino acid residues 590–831 (reviewed by Dahan et al., 2001; Aleksandrov et al., 2007). In human and killifish CFTR sequences there are approximately 20 PKA and protein kinase C (PKC) sites in the R domain. The disease cystic fibrosis often arises from mutations that interfere with the trafficking of CFTR product into the plasma membrane, of which the delta F508 deletion is the most common (Aleksandrov et al., 2007). The disease progresses often to chronic lung infection, cystic lesions and ultimately death. There is another somewhat more rare and less severe type of manifestation of the disease, one involving the normal insertion of the CFTR protein in the plasma membrane but still abnormal operation because of a failure of the ion channel to be fully activated. This form may result from inadequate phosphorylation of the regulatory domain for activation of the channel. The impediment of cAMP mediated activation suggests that this form involves a phosphorylation activation pathway.

Teleost fish possess CFTR in the apical membrane of mitochondria rich salt transporting (MR) cells in the gills and opercular membrane, which are responsible for salt secretion and successful acclimation of marine fish to seawater and also for acclimation of hardy euryhaline species, such as *Fundulus heteroclitus* (Griffith, 1974; Hoffmann et al., 2002; Zadunaisky et al., 1995), to hypersaline conditions. CFTR has been cloned and sequenced from killifish gill and is a divergent homologue of the mammalian version of the gene (Singer et al., 1998). The lack of other identifiable anion channels and insensitivity of chloride secretion to the disulphonic stilbene DIDS point to CFTR as the cAMP activated anion channel of MR cells in teleost fish (Marshall et al., 1995). The channel is activated by cAMP and PKA (Marshall et al., 1995; Singer et al., 1998), as is true for mammalian systems (Aleksandrov et al., 2007). Most phosphorylation sites of human and teleost CFTR are conserved, such that the regulation and activation of CFTR in teleosts is in many ways similar to that in mammals. Euryhaline teleosts are distinct in that CFTR, which is usually expressed in a static 'housekeeping' fashion in mammalian tissues, can be induced to increase expression by simple transfer of the animal from dilute salinity to seawater or higher salinities (Singer et al., 1998). Thus regulation of CFTR expression and its plasticity is easily studied in the teleost model system. Furthermore, teleostean CFTR can be rapidly deactivated and activated through manipulation of neurotransmitters, hormones and medium osmolality. In this way, the euryhaline teleost *F. heteroclitus*, a well known and intensely studied physiological and genomic model of salt transport (Burnett et al., 2007), provides a unique opportunity to study the regulation of this clinically important regulatory ion channel.

CFTR regulation has been well studied, yet the means by which phosphorylation of the R domain occurs is still not well understood (Alexsandrov et al., 2007). R domain phosphorylation is essential

for channel gating, and ATP binding in the two nucleotide binding domains (NBD1 and 2) is an essential prerequisite as well. There is a PDZ binding domain (–DTRL) at the carboxyterminus of CFTR that is conserved from fish to human (Singer et al., 1998) and may function in channel gating as well as membrane insertion of CFTR (Li et al., 2005). CFTR also is aided by serum and glucocorticoid activated kinase (SGK1) in mammalian systems and in killifish (Shaw et al., 2008; Sato et al., 2007). Although regulatory complexes involving CFTR have thus been identified (Naren et al., 2003; Li et al., 2005), none has connected CFTR with osmosensing systems or with tyrosine kinase activation, yet tyrosine kinase sites exist in the R domain of CFTR.

For this reason, we embarked on studies to examine the feasibility of tyrosine phosphorylation of the regulatory domain being a means of CFTR channel activation. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that generally resides in focal adhesions (Tani et al., 1996), is associated with cell motility (Parsons, 2003) and invasive cancer (Owens et al., 1995), possesses a protein 4.1, ezrin–radixin–moesin (FERM) domain (Parsons, 2003) and may be phosphorylated at tyrosines Y397, Y407, Y576, Y577, Y861 and Y925 (Parsons, 2003). Tyrosine 407 and the immediate C-terminal flanking residues (Y407-T-M-P) are conserved among FAK sequences from diverse vertebrates (Hanks and Polte, 1997). However, FAK Y407 phosphorylation function remains unclear. In mammalian fibroblasts, phosphorylation of FAK at Y407 is associated with contact inhibition (Lim et al., 2007). Hyperosmotic shock activates FAK, producing phosphorylation at Y397 and Y577 in several different mammalian cell lines (Lunn and Rozengurt, 2004) and FAK is thought to regulate osmotic responses (reviewed by Hoffmann et al., 2009).

In preliminary studies we observed that CFTR is present in the apical membrane of seawater MR cells in a variety of teleost fish gills and skin [mudskipper (Wilson et al., 2000); killifish (Marshall et al., 2002); Hawaiian goby (McCormick et al., 2003); European eel (Wilson et al., 2004); and tilapia (Hiroi et al., 2005)]. In previous immunocytochemical experiments we discovered a novel association of CFTR and the tyrosine kinase FAK (Marshall et al., 2008). Using phosphorylation specific antibodies directed to conserved tyrosine phosphorylation sites on FAK, we localized FAK pY407 and colocalized FAK pY576 with CFTR in the apical membranes of MR cells (Marshall et al., 2008). Furthermore, we observed that other stimuli that inhibit Cl^- secretion by the MR cells also dephosphorylate FAK pY407, thus preventing FAK immunostaining at pY407 (Marshall et al., 2008). Hence, there is a potential functional relationship between CFTR and FAK in the apical membrane of MR cells.

The present study expands on these preliminary experiments to start to reveal the relationship between FAK phosphorylation and CFTR activation. This was done by extending previous findings to include immuno-transmission electron microscopy (TEM) experiments to pinpoint their colocalization, by experimentally relating stimulation of previously inhibited membranes to the rephosphorylation of FAK (i.e. reversibility) and to establish the phosphorylation/activation of CFTR *via* tyrosine phosphorylation.

MATERIALS AND METHODS

Animals

Adult killifish (*Fundulus heteroclitus* L.) of both sexes were obtained from the Antigonish estuary (Nova Scotia, Canada), transferred to indoor holding facilities and kept in full strength seawater with salinity 32 g l^{-1} at 17–21°C and ambient photoperiod under artificial light. 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}, supplemented three times weekly with mealworms (*Tenebrio molitor*). Killifish were netted and killed by decapitation prior to the experiments. Fish opercula (gill covers) were cut off and pinned on a cork platform placed under a dissecting microscope. Gill arches were removed and opercular epithelia were dissected by carefully removing the attached membrane from the opercular bone.

Bathing solutions

Control membranes that received isotonic treatment were incubated in 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, 315 mosmol kg^{-1}, when equilibrated with a 99% O_2/1% CO_2 gas mixture) before being fixed. Test membranes that received hypotonic shock treatment were flushed with a diluted 80% Cortland's saline and continuously aerated with a 99% O_2/1% CO_2 gas mixture to maintain pH balance of the solutions. Test membranes that received hypertonic post-treatments were incubated with a higher osmolality Cortland's saline (375 mosmol kg^{-1}) in which NaCl content was increased (to 202 mmol l^{-1}).

Antibodies

The primary antibodies used to detect FAK phosphorylated on tyrosine residues were rabbit polyclonal anti-human FAK pY407 and FAK pY576 immunopurified against the epitope (Biosource, Camarillo, CA, USA). The phosphorylated tyrosine epitope regions of FAK corresponding to this antibody are known to be highly conserved between human (GenBank accession no. L05186) and pufferfish *Takifugu rubripes* (Blast search on *Fugu* genomics project website: http://www.fugu-sg.org/). The primary antibody used for detection of killifish CFTR was mouse monoclonal anti-human CFTR (R&D Systems, Minneapolis, MN, USA) directed against the conserved carboxyterminal domain in killifish (Singer et al., 1998), thus the antibody is selective for this protein (Marshall et al., 2002). The secondary antibodies used for immunofluorescence microscopy were either goat anti-mouse IgG or goat anti-rabbit IgG polyclonal conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA). Secondary antibodies used for immunogold labelling were goat anti-mouse IgG conjugated with 15 nm gold and goat anti-rabbit IgG conjugated with 10 nm gold (Electron Microscopy Sciences, PA, USA).

Immunocytochemistry

The opercular epithelia were dissected without the dermal chromatophore layer and pinned to modeller's wax. The membranes received each treatment on the wax and were then fixed for 2 h at –20°C in 80% methanol/20% dimethylsulphoxide (DMSO). Following fixation, they were rinsed three times in rinsing buffer comprising 0.1% bovine serum albumin (BSA) in 0.05% Tween 20 in phosphate-buffered saline (TPBS; composition in mmol l^{-1}: NaCl 137, KCl 2.7, Na_2HPO_4 4.3 and KH_2PO_4 1.4 at pH 7.4), then immersed in a blocking solution with 5% normal goat serum (NGS), 0.1% BSA, 0.2% NaN_3 in TPBS, pH 7.4 for 30 min at room temperature in the dark and finally incubated with each primary antibody (10 μg ml^{-1} in blocking solution), singly and in combination, at 4°C overnight. Control and test membranes were then rinsed three times and exposed to the secondary antibodies (8 μg ml^{-1} in blocking solution), singly and in combination, for 4 h at room temperature. After three final rinses the membranes were mounted in mounting medium (Geltol; Immunon Thermo Shandon,

Pittsburgh, PA, USA). Slides were viewed and images were collected with a laser scanning confocal microscope (Olympus, Markham, ON, Canada; model FV300). In each opercular membrane, randomly selected *Z*-stack series were collected using a ×40 water objective (NA 1.15), zoom of 3.0 and with optical sections of 1.0±0.05 μm. An average of 35 sections was collected for each image. *XZ* scans were obtained automatically by setting an observation line through the apical crypt of each cell. Line scans were obtained by scanning the membranes in *XYZ* mode first and then drawing a straight line across the centre of the apical crypt of a given cell. The system scanned the line over the focus plane of the cell along the *Z*-axis. The software then automatically measured the intensity and distribution differences of all fluorescence channels. The final fluorescence intensities were given in arbitrary units *versus* the distance in μm from the original line drawn across the cell.

Control membranes under isotonic conditions were simply incubated for 60 min in modified Cortland's saline prior to fixation. This was done in parallel with the test membranes, which received a 60 min hypotonic shock treatment by being incubated in aerated 80% Cortland's saline.

For forskolin, isoproterenol and 3-isobutyl-1-methylxanthine (IBMX) post-treatments, both of the paired membranes received hypotonic shock pretreatment for 60 min at room temperature. Then one side of the pairs (control) was fixed and immunohistologically labelled as mentioned above. The other side of the pairs (test) was incubated in forskolin (10 μmol l^{-1}), isoproterenol (1 μmol l^{-1}) or IBMX (0.1 mmol l^{-1}) for 10 min at room temperature (all final concentrations, diluted in 80% hypotonic Cortland's saline).

For clonidine pretreatment, control membranes were incubated in aerated Cortland's saline with 10 μmol l^{-1} clonidine. Test membranes were post-incubated in hypertonic Cortland's saline for 10 min at room temperature.

Pharmaceuticals

The cAMP activators forskolin and isoproterenol (Sigma-Aldrich, St Louis, MO, USA) stock solutions were dissolved in Cortland's saline. IBMX (Sigma-Aldrich) stock solution was dissolved in ethanol, which had no effect on the electrophysiological variables. The α_2-adrenoceptor agonist clonidine hydrochloride (Sigma-Aldrich) stock solution was also dissolved in saline.

Immunogold TEM

Opercular membranes from seawater killifish were fixed in 0.2% glutaraldehyde–2% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (PB, pH 7.4) for 3 h. After dehydration in ethanol, the membrane tissues were embedded in LR white resin (London Resin Company, London, UK) and ultrathin sections (80 nm) were cut with a diamond knife. The ultrathin sections on a nickel grid were incubated sequentially with (1) blocking solution (2% NGS, 0.1% BSA, 0.05% Triton X-100, 0.01% NaN_3 in PBS) for 10 min, (2) primary antibodies (anti-hCFTR and anti-FAK pY407, 20 μg ml^{-1}) diluted in PBS at 4°C overnight, (3) immunogold secondary antibodies diluted 1:50 in PBS for 2 h at room temperature, followed by five rinses in PBS for 5 min each. The immunolabelled sections were then washed with filtered water, stained with uranyl acetate for 3 min and lead citrate for 2 min, and viewed using a TEM (Philips EM 410, Eindhoven, The Netherlands).

Protein extractions

Opercular membranes, gill filaments and heart tissue from 26 killifish were pooled and treated in 10 ml isotonic Cortland's saline, hypotonic solution, hypotonic solution followed by forskolin addition (10 μmol l^{-1}) or hypotonic solution followed by hypertonic shock, aerated with a 99% O_2/1% CO_2 gas mixture. Membranes were then homogenized in 4 ml of ice cold SEI buffer (300 mmol l^{-1} sucrose, 20 mmol l^{-1} EDTA, 100 mmol l^{-1} imidazole) using a Polytron homogenizer. The treated extracts were centrifuged at 2000 ***g*** for 5 min at 4°C. Pellets were resuspended and rehomogenized in 1 ml of 2.4 mmol l^{-1} deoxycholic acid in SEI buffer. The samples were centrifuged at 2000 ***g*** for 5 min at 4°C and the supernatant (crude protein) was collected. The protein concentration of each sample was determined using the Bradford method (Bradford, 1976). The extracted crude proteins (30 μg) were denatured in lane marker sample buffer (Thermo Scientific, Rockford, IL, USA). Positive controls were performed using killifish gill, a tissue known to express CFTR through its MR cells. Negative controls were performed using killifish heart, a tissue lacking CFTR expression (Singer et al., 1998).

Co-immunoprecipitations

Co-immunoprecipitations were performed by incubating 250 μg of crude protein with 5 μl of each primary antibody, overnight at 4°C, under gentle rotation followed by the addition of a 50% slurry of 20 μl protein A–Sepharose (4B Fast Flow, Sigma-Aldrich) with an overnight incubation at 4°C. The immunoprecipitate–Sepharose bead complexes were pelleted, washed 5 times in SEI, resuspended in sample buffer and boiled for 5 min. The immunoprecipitated proteins were recovered after centrifugation at 5000 ***g*** for 3 min. Positive and negative controls were performed in order to confirm the specificity of the immunoprecipitations. Membranes were stained for protein (Ponceau S) as a check against non-specific protein precipitation.

Western blotting

Proteins were separated by SDS-PAGE (precast 10% Tris-HCl Precise™ protein gels, Thermo Scientific) and electro-transferred to Immobilon-P membranes (Millipore, Bedford, MA, Canada). Protein band sizes were determined using Kaleidoscope standards (Bio-Rad laboratories, Mississauga, ON, Canada). After the transfer, the blots were incubated in blocking buffer (Tris-buffered saline plus 0.05% Tween 20, TTBS; composition in mmol l^{-1}: Tris-HCl 20, NaCl 500, KCl 5, pH 7.5), supplemented with 3% BSA for 4 h at room temperature. Both CFTR and FAK pY407 primary antibodies were diluted 1:1000 and applied overnight at 4°C. The membranes were then washed three times in TTBS for 5 min each. The anti-mouse and anti-rabbit, alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich) were diluted 1:500 in blocking buffer and applied for 2 h at room temperature. The membranes were washed again and visualized using 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) membrane phosphate substrate (Roche Diagnostics, Indianapolis, IN, USA). The membranes were then reprobed with anti-actin polyclonal antibody (1:200; Sigma-Aldrich) and the intensity of the immunoreactive bands was quantified by densitometric scanning and normalized with respect to actin. Densitometric quantification of western blots was performed using an HP PSC 2400 scanner (Hewlett Packard, Oceanside, CA, USA) and UN-SCAN-IT gel version 5.1 software for Windows (Silk Scientific, Orem, UT, USA). Background of each image was subtracted by selecting a square of background with no specific bands.

Electrophysiology

The opercular epithelium was removed without the nerve supply and mounted in an Ussing chamber as described previously

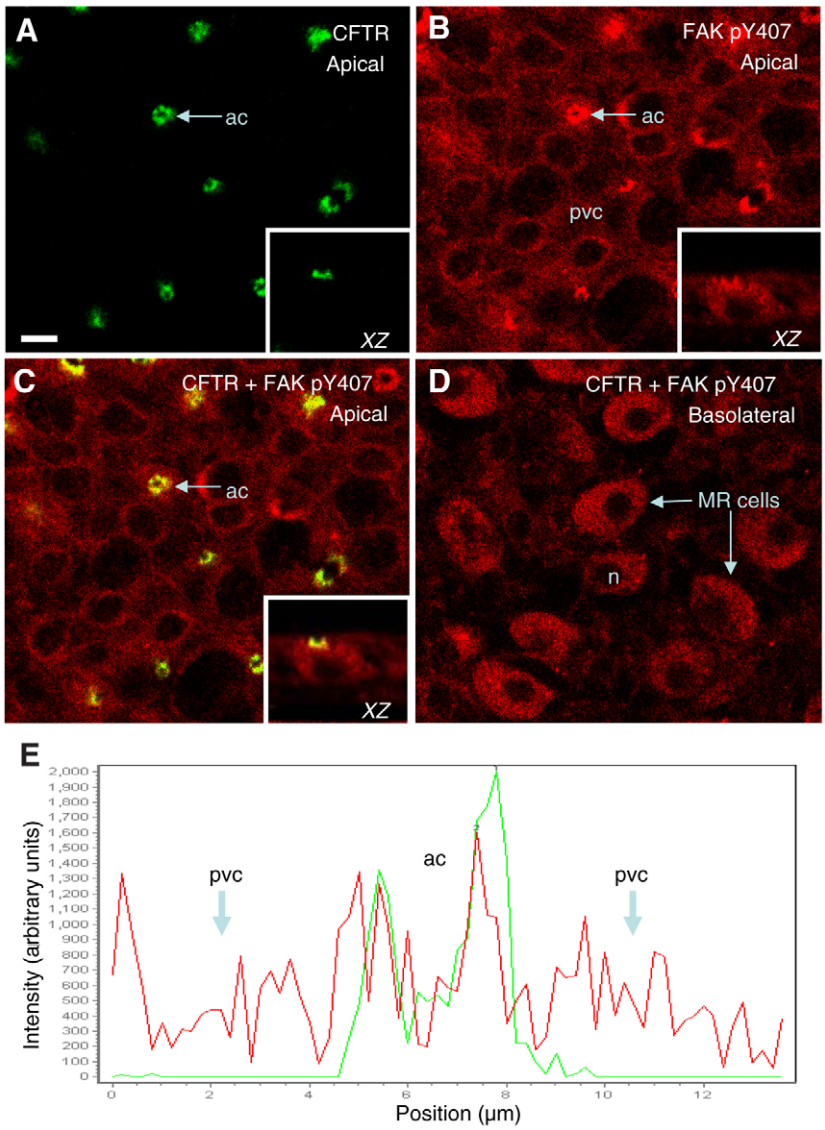

Fig. 1. Immunocytochemistry for cystic fibrosis transmembrane conductance regulator (CFTR) and focal adhesion kinase (FAK) pY407 [primary antibodies: mouse monoclonal anti-human CFTR and rabbit anti-phosphorylated human FAK pY407; secondary antibodies: goat anti-mouse IgG Alexa Fluor 488 (green) and goat anti-rabbit IgG Alexa Fluor 594 (red)]. (A) CFTR immunofluorescence was present in the apical crypt of mitochondria rich (MR) cells (green). The inset shows an *XZ* view of CFTR immunofluorescence in the apical crypt (*N*=12). (B) FAK pY407 immunofluorescence was present in the pavement cells and in the apical crypt of MR cells (red). The inset shows an *XZ* view of FAK pY407 immunofluorescence in the apical crypt (*N*=12). (C) There was a high degree of colocalization of CFTR and FAK pY407 in the apical crypt (yellow). The inset shows an *XZ* view of CFTR and FAK pY407 immunofluorescence in the apical crypt (*N*=12). (D) Same frame as C, but 10 µm below. At this level only FAK pY407 was present. (E) A line scan of fluorescence intensity (arbitrary units) *versus* distance in µm across the apical crypt of a double labelled MR cell showing good correspondence in the colocalization of CFTR (green line) and FAK pY407 (red line) in the crypt periphery. ac, apical crypt; n, nucleus; pvc, pavement cells; MR cells, mitochondria rich cells. Scale bar, 10 µm.

(Marshall et al., 1999). 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 ($\Omega\,cm^2$) 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). A current–voltage clamp (D. Lee, Sunnyvale, CA, USA) was used to measure the epithelial variables.

To test the effects of forskolin, isoproterenol and IBMX on the Cl^- secretion by MR cells in killifish opercular membranes, I_{sc} of the test membrane was monitored parallel to the paired control membrane from the same animal. After a period of at least 30 min to establish the resting I_{sc} of the membrane in isotonic saline on both the serosal and mucosal sides, the serosal side of test membranes received either of following 60 min treatments; (i) 10 $\mu mol\,l^{-1}$ of forskolin, 1 $\mu mol\,l^{-1}$ of isoproterenol or 0.1 $mmol\,l^{-1}$ of IBMX by addition of the stock solutions mentioned above, or (ii) 80% hypotonic Cortland's saline (60 min) followed by the addition of forskolin, isoproterenol or IBMX (same concentrations as for i). The paired control membranes stayed in isotonic saline for i, and received only 80% hypotonic saline treatment for ii.

To test the effects of clonidine and hypertonicity, clonidine (10 $\mu mol\,l^{-1}$) was added to the serosal side of the control membranes (60 min). For the test membranes, hypertonic or isotonic saline containing clonidine was applied to the serosal side by the flow through method. I_{sc} was not permitted to reach steady state between the two additions.

In parallel, the effects of hypotonic shock alone followed by hypertonic flushes (no added clonidine) were also tested.

Statistical analysis

Data are expressed as means ± 1 s.e.m. Statistical analyses performed on the immunoblot results (quantitative assessments) were determined by single classification analysis of variance (ANOVA) followed by Bonferroni's *a posteriori* comparisons test. Transport activation comparisons as well as differences in the effects of hypotonicity, clonidine and hypertonicity on opercular membranes were performed using paired *t*-tests between test and control membranes and single classification ANOVA followed by Bonferroni's *a posteriori* comparisons test for differences between groups. Statistical significance was ascribed if $P<0.05$.

RESULTS

CFTR and FAK pY407 distribution in isotonic conditions

We previously reported that CFTR distribution in seawater killifish opercular epithelia was found exclusively in the apical region of MR cells (Marshall et al., 2002). In the present study, immunocytochemistry revealed positive staining for both CFTR (green) and FAK pY407 (red, Fig. 1A,B). In isotonic conditions, CFTR immunolocalization was restricted to the apical region and found solely in the apical crypt of MR cells (Fig. 1A). FAK pY407 immunofluorescence, on the other hand, was evenly distributed throughout the MR cells. This protein was present in the pavement cells, in the apical crypt and in the basolateral membrane tubular system of the MR cells (Fig. 1B,D). Apically located FAK pY407 was found to be colocalized with the CFTR signal (yellow) shown in the *XZ* scan (insets of Fig. 1A,B,C) and line scan obtained by confocal laser scanning microscopy (Fig. 1C,E). The cup shape in the figures (Fig. 1C) is typical of the concave shape of the apical crypt as viewed from the side.

The distribution of CFTR and FAK pY407 in the MR cells of saltwater killifish was also determined by immunogold double-labelling using TEM (Fig. 2A). FAK pY407 and CFTR immunogold particles were closely distributed and colocalized in the apical crypt of the MR cells. Furthermore, CFTR and FAK pY407 were colocalized in membrane bound vesicles subjacent to the apical membrane (Fig. 2C,D). However, only FAK pY407 immunogold particles were present throughout the basolateral membrane (Fig. 2E), a confirmation of previous immunoelectron microscopy findings (Marshall et al., 2008). Interestingly, FAK pY407 was also detected in the pavement cells (Fig. 2B), in the absence of CFTR. These results coincided with our immunocytochemistry findings (Fig. 1) and therefore confirmed the close distribution of the two proteins in the apical crypt region and the absence of CFTR in the pavement cells and in the basolateral membrane tubular system.

Effects of treatments on the protein localizations

As previously demonstrated (Marshall et al., 2008) following 80% hypotonic pretreatment (60 min) to isolated killifish opercular epithelia, FAK pY407 became dephosphorylated in the basolateral region of the MR cells. However, FAK activity in granular lymphocytes (interspersed among epithelial cells) proved to be insensitive to hypotonic shock (Fig. 3A,B). Previous data acquired

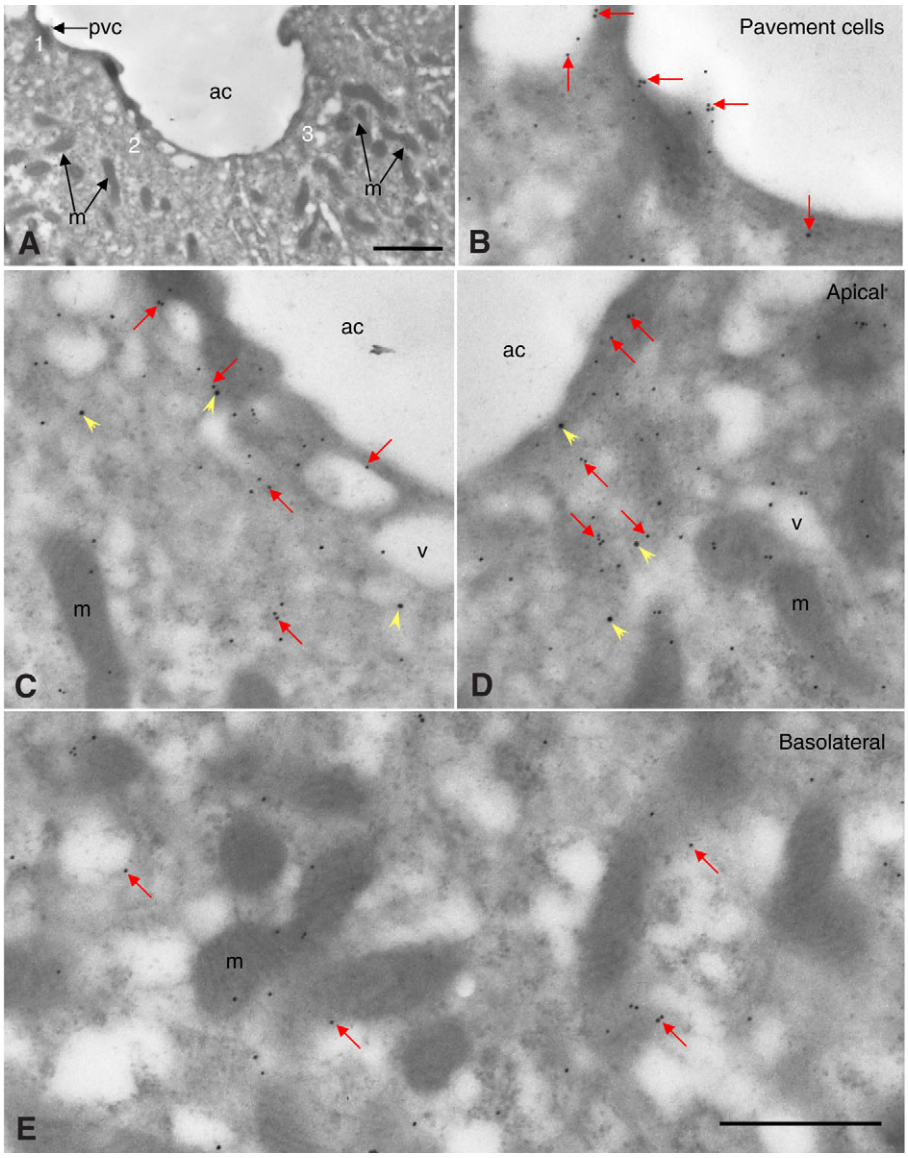

Fig. 2. Transmission electron micrographs of immunogold double labelling using CFTR (primary: mouse monoclonal anti-human CFTR; secondary: goat anti-mouse IgG–15 nm gold) and FAK pY407 (primary: rabbit anti-phosphorylated human FAK pY407; secondary: goat anti-rabbit IgG–10 nm gold) (*N*=7). (A) Low magnification micrograph of an MR cell. Scale bar, 1 μm. (B) Magnified view of region 1 of A showing the presence of FAK pY407 only (red arrows) in the pavement cells. (C) Both CFTR and FAK pY407 were detected in the apical region of MR cells. A magnified view of the apical crypt (region 2 of A) showed CFTR (yellow arrowheads) and FAK pY407 colocalization. (D) FAK pY407 was also present in apical membrane and subapical vesicles of the MR cells (region 3 of A). There was a close distribution of the two proteins along the apical crypt edge of the MR cells. CFTR was also found in the subapical vesicles (not shown). (E) Double labelling in the cytoplasmic area of the cells showed only FAK pY407 in the basolateral membrane. ac, apical crypt; m, mitochondria; pvc, pavement cells; v, vesicle. Scale bar for B–E, 0.5 μm.

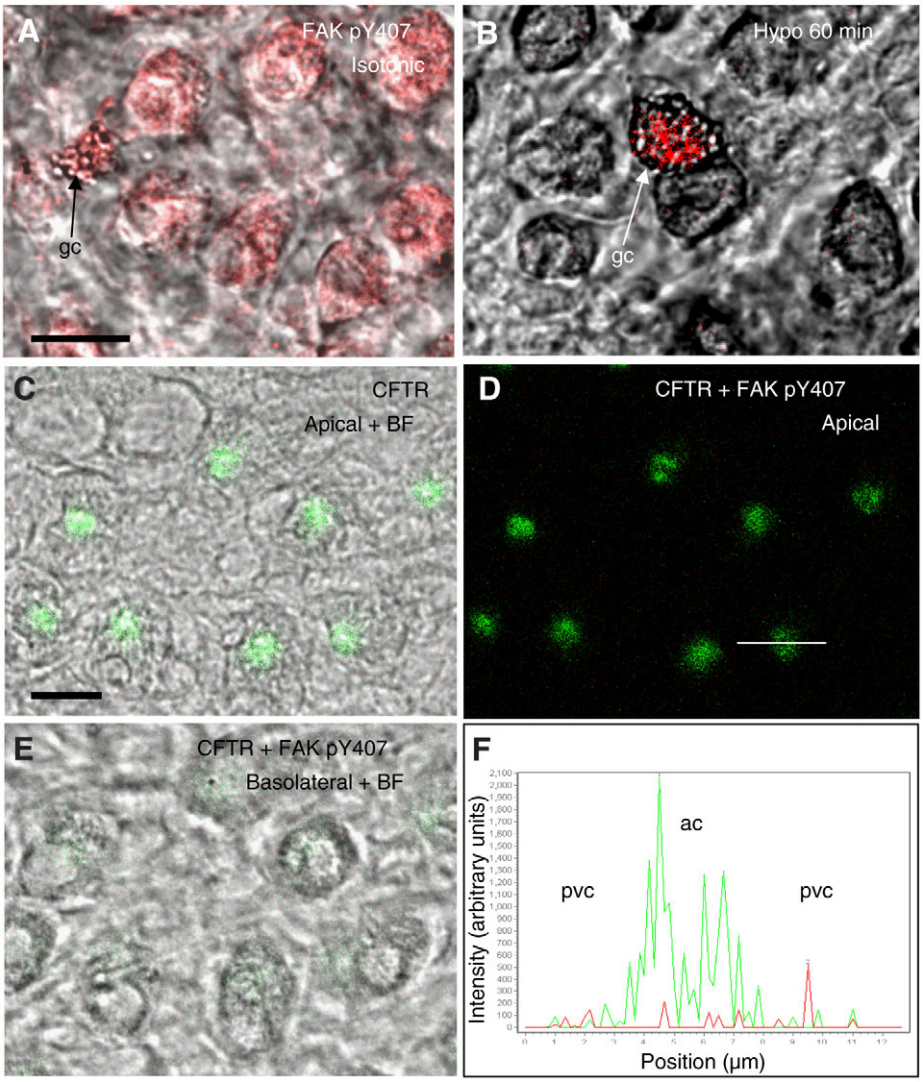

Fig. 3. Immunocytochemistry for CFTR (green) and FAK pY407 (red) following hypotonic shock pretreatment. (A) In isotonic conditions, FAK pY407 immunofluorescence, shown in bright field (BF), was present in the basolateral membrane (*N*=12). (B) Basolateral FAK pY407 immunofluorescence was eliminated following a 60 min hypotonic shock pretreatment (*N*=12). FAK in granular lymphocytes was insensitive to hypotonic shock. (C) CFTR immunofluorescence was still present in the apical crypt following hypotonic shock (*N*=12). (D) Same frame as C, showing the absence of FAK pY407 in the pavement cells and in the apical crypt. (E) Same frame as C, but an optical section 10 µm below. Neither CFTR nor FAK pY407 was apparent at this level, which we interpret as the absence of CFTR and dephosphorylation of FAK. (F) A line scan of fluorescence intensity (arbitrary units) *versus* distance in µm across the apical crypt of a double labelled MR cell (indicated by the white line in D) confirmed the presence of CFTR fluorescence (green line) in the apical crypt and the absence of FAK pY407 fluorescence (red line) in that same area and in the pavement cells. gc, granular lymphocytes; ac, apical crypt; pvc, pavement cells. Scale bar for A and B, and C–E, 10 µm.

by TEM, confocal microscopy and differential interference contrast (W.S.M., K.D.W., L.R.H., R.R.F.C. and F.K., unpublished data) allowed us to visually identify the granular lymphocytes on the basis of their characteristics (Barnett et al., 1996; Holland and Rowley, 1998). As expected, CFTR immunofluorescence was still present in the apical crypt (Fig. 3C), but FAK pY407 fluorescence was absent from apical crypts, pavement cells (Fig. 3D) and central locations (Fig. 3E). FAK pY407 dephosphorylation of the entire MR cells was shown by the absence of red fluorescence in the line scan across an apical crypt (Fig. 3F).

After the addition of the cAMP activators forskolin, isoproterenol and IBMX to the opercular epithelia (Fig. 4A,D,G respectively) for 10 min following 60 min of hypotonic shock, dephosphorylated FAK at pY407 became strongly rephosphorylated in the apical membrane of MR cells and, based on fluorescence intensity, considerably less so in the subapical and basolateral locations in the MR cells (compare *XZ* scans of control, Fig. 1B, and rephosphorylated FAK, Fig. 4A). As expected, CFTR distribution remained unchanged following the addition of the drugs (Fig. 4B,E,H). Merged immunofluorescence confirmed a high degree of colocalization between CFTR and FAK pY407 in the apical crypt, and the absence of phosphorylated FAK in the rest of the cell (Fig. 4C,F,I).

Clonidine pretreatment for 60 min had no effect on CFTR distribution; immunofluorescence was limited to the apical crypt of the MR cells (Fig. 5A,B). However, clonidine incubation dephosphorylated FAK at pY407 in the pavement cells, apical crypt and basolateral membrane (Fig. 5C). Parallel run isotonic controls (not shown) and hypertonic post-treatment of clonidine-pretreated opercular epithelia also had no effect on CFTR immunostaining (Fig. 5D). However, isotonic solutions (not shown) and hypertonicity following clonidine incubation rephosphorylated FAK at pY407. As with forskolin and IBMX post-treatment, the immunofluorescence signal was restored most strongly in the apical crypt (Fig. 5E). Furthermore, merged immunofluorescence detected the colocalization of CFTR and newly rephosphorylated FAK in the apical crypt of MR cells (Fig. 5F).

CFTR and FAK pY576 distribution

As previously reported (Marshall et al., 2008), FAK pY576 distribution was found exclusively in the apical crypt of MR cells and in this study we found that FAK pY576 colocalized with CFTR under isotonic conditions (Fig. 6A–D). Immunofluorescence for FAK pY576 and colocalization with CFTR, shown by means of line scans, remained strongly positive following hypotonic pretreatment (Fig. 6E,F), hypotonic pretreatment followed by

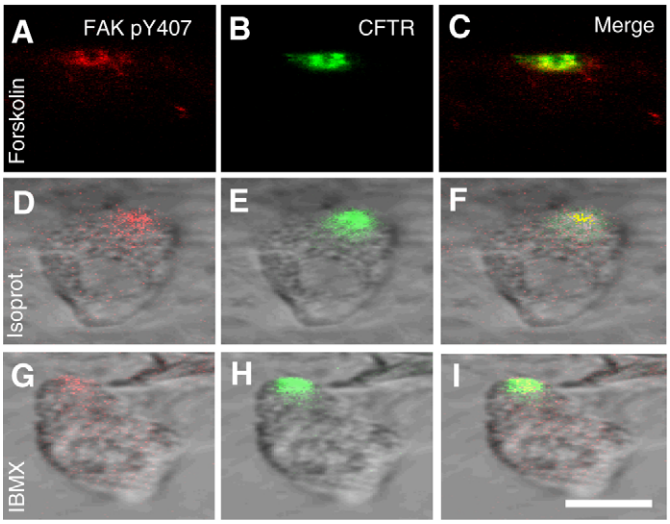

Fig. 4. Immunocytochemistry for CFTR (green) and FAK pY407 (red) following 60 min hypotonic shock pretreatment and 10 min post-treatment with cAMP activators forskolin (10 μmol l^{-1}, *N*=9), isoproterenol (1 μmol l^{-1}, *N*=6) and 3-isobutyl-1-methylxanthine (IBMX, 0.1 mmol l^{-1}, *N*=6). Shown here in *XZ* views, FAK pY407 immunofluorescence was restored in the apical crypt and partly in the subapical region following the addition of forskolin (A), isoproterenol (D) and IBMX (G). CFTR distribution was unchanged following the addition of all three cAMP activators (B, E, H). Merged immunofluorescence showed a high degree of colocalization between FAK pY407 and CFTR in the apical crypts (C, F, I). Scale bar, 10 μm.

forskolin addition (Fig. 6G,H) and hypotonic pretreatment followed by hypertonic shock (Fig. 6I,J).

Protein expression

We measured CFTR, FAK pY407 and FAK pY576 activation in pretreated opercular membranes by western blot analyses. The antibody specific to CFTR detected a band at ~140 kDa. The resulting immunoreactivity was the same for all treatments (Fig. 7A) and CFTR expression level was similar in all treated and non-treated groups (Fig. 7D).

The antibody specific to FAK pY407 detected a strong band at ~135 kDa. FAK pY407 immunoreactivity was stronger in isotonic treatments and in hypotonic treatments if they were followed by forskolin addition or hypertonic shock. FAK pY407 dephosphorylation brought on by hypotonic shock alone revealed a weaker band (Fig. 7B, top panel). However, when immunoblots were reprobed with anti-FAK pY576, immunoreactivity was similar for all treatments (Fig. 7B, middle panel). As previously reported (Marshall et al., 2008), FAK pY576 proved to be insensitive to hypotonic shock.

Gill tissue was used as a positive control and heart tissue as a negative control for all three antibodies. The presence of CFTR, FAK pY407 and FAK pY576 in killifish gill was confirmed by a single visible band specific to each protein (~140 kDa for the CFTR and ~135 kDa for the two FAK phospho-antibodies). Neither CFTR nor phosphorylated FAK was detected in heart tissue, the negative control (Fig. 7C).

Western blot quantitative analysis revealed that CFTR protein expression remained at the same level following all treatments and that there was no significant difference in protein expression levels between the controls and the treated groups for FAK pY576. However, FAK pY407 protein expression in hypotonic conditions was 62%, significantly lower than that of the control group. These results confirm our previous findings, where hypotonic shock inhibited chloride secretion (e.g. Marshall et al., 2000) in parallel with decreased FAK pY407 levels in opercular epithelia (Marshall et al., 2008). Furthermore, FAK pY407 phosphoprotein expression was restored following forskolin addition (to 86% of control) and hypertonic shock post-treatments (to 89%, Fig. 7D).

In order to confirm the relationship between CFTR and FAK pY407, we isolated by immunoprecipitation both proteins from killifish opercular epithelia. As expected, we found that CFTR co-immunoprecipitated with FAK pY407 (Fig. 8, lanes 2 and 3). CFTR and FAK pY407 positive controls revealed specific bands at ~140 kDa and ~135 kDa, respectively (Fig. 8, lanes 1 and 4). These experiments were done using the same antibody during the immunoprecipitation and blotting steps. Negative controls, where the primary antibodies were omitted during the immunoprecipitation, did not reveal any visible bands (Fig. 8, lanes 5 and 6).

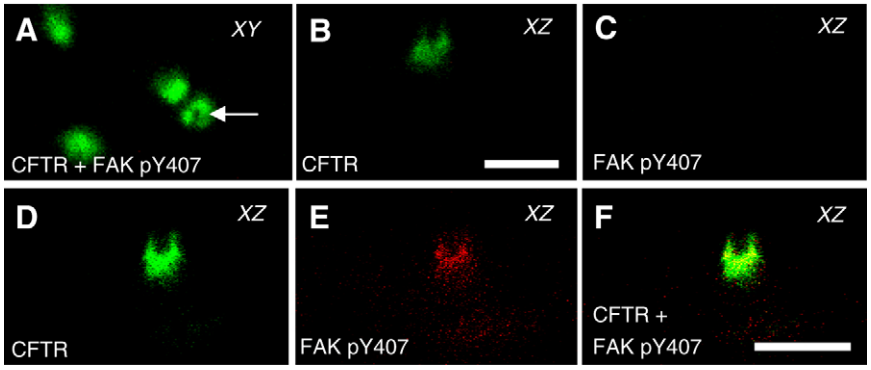

Fig. 5. Immunofluorescence for CFTR (green) and FAK pY407 (red) following 60 min clonidine (10 μmol l^{-1}) pretreatment and 10 min hypertonic post-treatment (*N*=11). Clonidine pretreatment had no effect on CFTR immunofluorescence in an *XY* scan at the level of the apical crypts (A). An *XZ* view of one MR cell (white arrow in A) showed positive staining for CFTR in the apical crypt (B). However, FAK pY407 immunofluorescence was completely eliminated following clonidine incubation in an *XZ* scan of the same cell (C). CFTR distribution remained the same following clonidine and hypertonic incubation (*XZ* scan of MR cell, D), while FAK pY407 immunofluorescence was restored in the apical crypt of the same cell (E). Merged immunofluorescence showed that the high degree of colocalization between FAK pY407 and CFTR in the apical crypts was also maintained under hypertonic conditions (F). Scale bars for A–C and D–F, 10 μm.

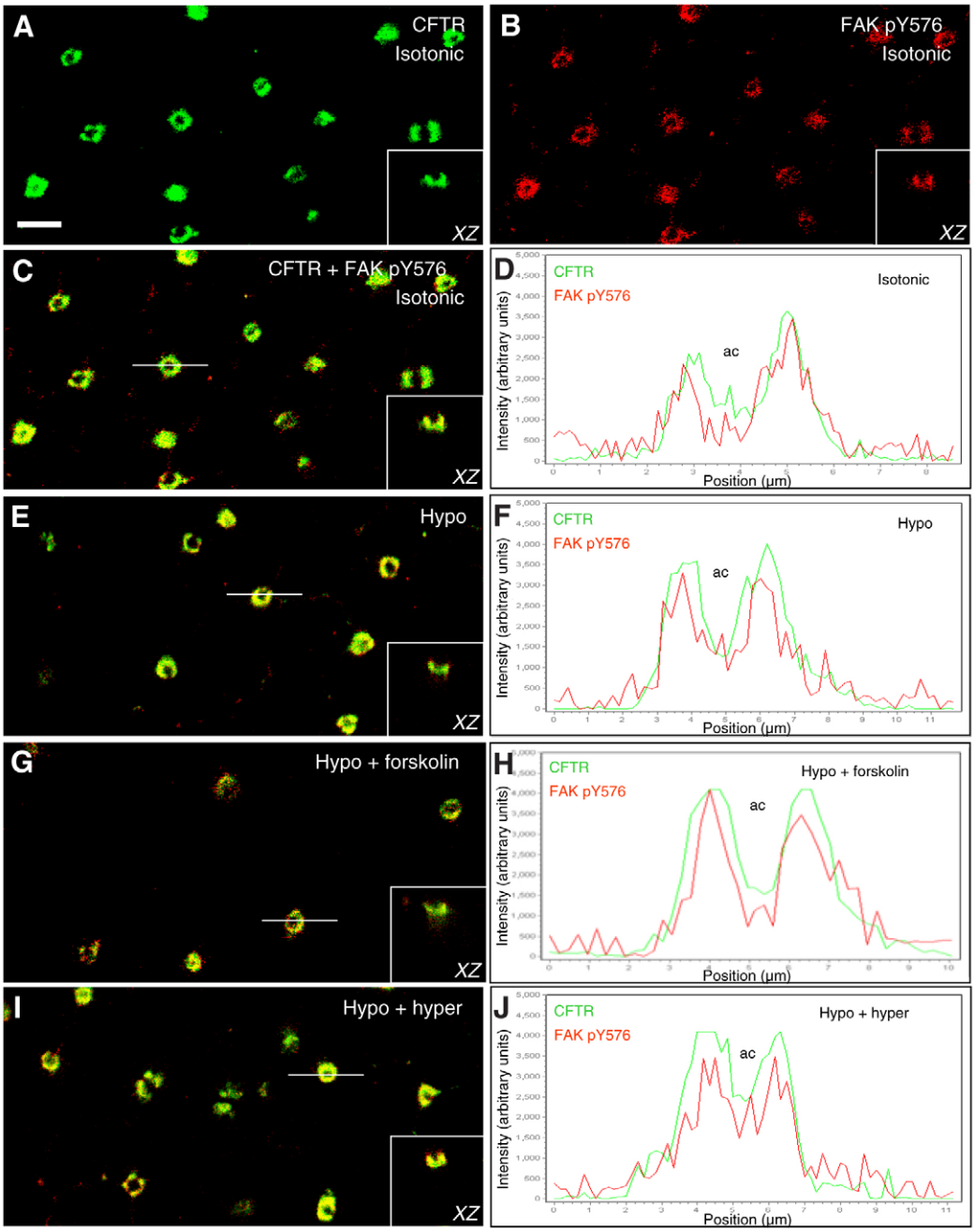

Fig. 6. Immunocytochemistry for CFTR and FAK pY576 [primary antibodies: mouse monoclonal anti-human CFTR and rabbit anti-phosphorylated human FAK pY576; secondary antibodies: goat anti-mouse IgG Alexa Fluor 488 (green) and goat anti-rabbit IgG Alexa Fluor 594 (red)]. In isotonic conditions, CFTR (A) and FAK pY576 immunofluorescence (B) were present in the apical crypt of MR cells. The insets show an *XZ* view of CFTR and FAK pY576 immunofluorescence in the apical crypt (*N*=12). (C) Merging panels A and B determined the colocalization of CFTR and FAK pY576 (yellow). The inset shows an *XZ* view of CFTR and FAK pY576 colocalization in the apical crypt (*N*=12). (D) A line scan of fluorescence intensity (arbitrary units) *versus* distance in µm across the apical crypt of a double labelled MR cell (indicated by the white line in C) showing a high degree of correspondence in the localization of CFTR (green line) and FAK pY576 (red line) in the crypt periphery following isotonic conditions. (E,F) CFTR and FAK pY576 distribution remained the same following 60 min hypotonic pretreatment (Hypo, *N*=6). (G,H) Hypotonic pretreatment for 60 min followed by forskolin addition, 10 min (Hypo + forskolin, *N*=6) and (I,J) 60 min hypotonic pretreatment followed by hypertonic shock, 10 min (Hypo + hyper, *N*=6). ac, apical crypt. Scale bar, 10 µm.

Electrophysiology

In the present study, we tested for Cl^- secretion rate electrophysiologically under the same conditions as we used for immunohistochemical experiments (above). When forskolin, IBMX and isoproterenol were added to the serosal side of control opercular membranes mounted in Ussing chambers filled with isotonic saline, there was a rapid increase in transepithelial current I_m (25%, 11% and 6%, respectively). However, this increase was only significant following the addition of forskolin compared with the initial value (Fig. 9A). An 80% hypotonic shock to the serosal side of the isolated epithelia mounted in Ussing chambers significantly decreased I_m by 51% compared with the isotonic condition. When activators were added to the same membranes on the serosal side in hypotonic saline, I_m was significantly recovered up to 84% (of control) with forskolin, 83% with IBMX and 100% with isoproterenol, compared with the initial isotonic value (Fig. 9B).

Although hypotonic treatment (60 min) significantly decreased I_m by 52%, hypertonic shock completely reversed the effect, followed by an overshoot of 56%. The α_2-adrenoceptor agonist clonidine had a stronger inhibitory effect on Cl^- secretion (82% decrease in I_m) compared with hypotonic treatment. Complete restoration of I_m by addition of isotonic and hypertonic solutions was not obtained. An isotonic solution rinse had no effect on clonidine inhibition and I_m levels remained significantly lower compared with the initial steady state of the membranes. The introduction of hypertonic solution in the presence of clonidine increased I_m by 27%, which was significantly higher than for clonidine alone but was lower compared with that of the membranes exposed to hypertonic conditions without clonidine (Fig. 10).

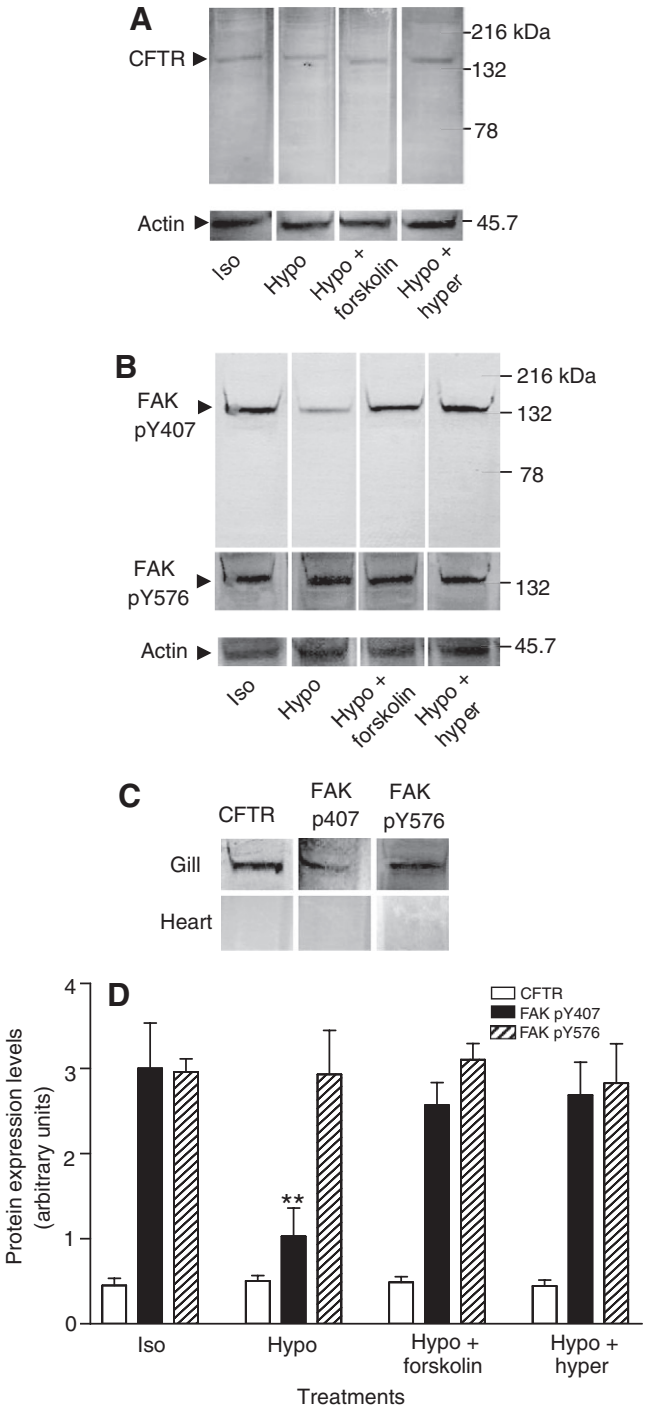

Fig. 7. Immunoblots of CFTR and FAK pY407 opercular epithelia under isotonic conditions (Iso, *N*=3), 60 min hypotonic pretreatment (Hypo, *N*=3), 60 min hypotonic pretreatment followed by forskolin addition, 10 min (Hypo + forskolin, *N*=3) and 60 min hypotonic pretreatment followed by hypertonic shock, 10 min (Hypo + hyper, *N*=3). (A) CFTR bands were detected at ~140 kDa in all treatments. (B) Strong bands were detected for FAK pY407 at ~135 kDa in isotonic conditions and in pretreated membranes with hypotonic + forskolin and hypotonic + hypertonic shock. A weaker band was detected following hypotonic pretreatment alone (top panel). Reprobing the immunoblot with FAK pY576 (middle panel) revealed visible bands at ~135 kDa for all treatments. A stronger band was detected (lane 2) following the hypotonic treatment compared with the previous immunoblot probed with FAK pY407. (C) CFTR, FAK pY407 and FAK pY576 were present in gill (positive control, *N*=3). Visible bands were detected at ~140 kDa (CFTR) and ~135 kDa (FAK pY407 and FAK pY576). Both CFTR and FAK proteins were absent from the heart tissue (negative control, *N*=3). (D) Quantitative western analysis of CFTR and FAK pY576 expression levels did not reveal any significant difference between the control and treated groups. However, FAK pY407 expression was significantly decreased after 60 min hypotonic shock compared with the control group, the hypotonic + forskolin treatment and the hypotonic + hypertonic treatment (**P<0.01). Statistical significance was determined by one way ANOVA followed by Bonferroni's post-test. The immunoreactive bands were quantified by densitometric scanning and normalized with respect to actin.

DISCUSSION

We previously demonstrated that tyrosine 407 was the only osmosensitive phosphorylation site in FAK protein and that FAK pY576, although insensitive to hypotonicity, colocalized with CFTR in the apical membrane (Marshall et al., 2008). The most important finding of the present study is that CFTR and FAK pY407 colocalize (by light microscopy, TEM and co-immunoprecipitation techniques) in the apical membrane of salt secreting MR cells and the two proteins appear to be in a regulatory relationship, wherein FAK dephosphorylation, specifically at Y407 (detected by immunocytochemistry and western blot analysis) and not at the nearby site of FAK Y576, is functionally and reversibly linked to the rate of Cl^- secretion by the epithelium. The colocalizations at light microscopy and TEM levels use primary antibodies against specific phosphorylation sites on FAK, FAK pY407 and FAK pY576, that are immunopurified to remove activity against dephosphorylated forms and are monoclonal for purity. In addition, the epitopes for these antibodies are completely conserved between fish (genomic models of *Danio rerio* and *Takifugu rubripes*) and humans. Likewise the CFTR antibody is against the completely conserved carboxyterminus of the protein, a PDZ-binding domain common from killifish (kfCFTR) to mammals. The mutually supportive findings of colocalization by light and electron microscopy reinforce the relationship in tissue that is lightly fixed (light microscopy) or strongly fixed (TEM); the western analysis confirms further the antibody operation on denatured protein. Co-immunoprecipitation of CFTR and FAK pY407 further supports a close structural, possibly functional relationship between the two proteins. We are therefore confident that the close proximity of CFTR and FAK in the apical membrane and subapical vesicles is a consistent feature of this epithelium. Agents that augment Cl^- secretion by activation of CFTR ion channels *via* cAMP (isoproterenol, forskolin and IBMX) phosphorylate FAK at position 407, while simultaneously activating NaCl secretion. Likewise, hypotonicity dephosphorylates and hypertonicity rephosphorylates FAK Y407 in parallel with Cl^- secretion rate, but without affecting cAMP. Whilst FAK phosphorylated at position 576 also colocalizes with CFTR, this phosphoprotein is insensitive to osmotic and cAMP mediated stimuli.

Osmosensitivity of MR cells

Euryhaline teleosts have relatively constant blood osmolalities, but during the acclimation process to salinity change, blood osmolality changes by 50–70 mosmol kg^{-1}. On entry into freshwater, such as normally occurs when these fish move into the freshwater lens to feed, blood osmolality can decrease significantly (Marshall et al., 2000; Marshall, 2003). Because the salt secretion is normally turned on in seawater animals, the physiologically relevant response is an inhibition on entry into dilute environments. Isolated opercular membranes exposed to hypotonic conditions immediately respond

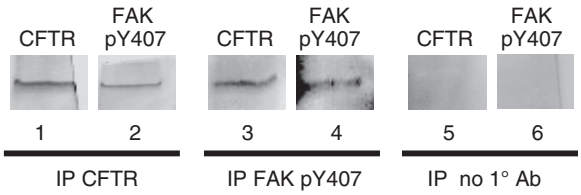

Fig. 8. Co-immunoprecipitation of CFTR with FAK pY407 in killifish opercular epithelium (N=3). Proteins immunoprecipitated (IP) with CFTR and blotted using anti-FAK pY407 revealed a band at ~140 kDa (lane 2). Similarly, co-immunoprecipitation of FAK pY407 with CFTR (IP with FAK pY407 and blotted using anti-hCFTR) revealed a band of the same molecular mass (lane 3). Positive controls for CFTR (IP with CFTR and blotted using anti-hCFTR) and FAK pY407 (IP with FAK pY407 and blotted using anti-FAK pY407) showed bands at ~140 kDa and ~135 kDa, respectively (lanes 1 and 4). There was an absence of bands in the negative controls (IP without primary antibodies and blotted with anti-hCFTR or anti-FAK pY407, lanes 5 and 6).

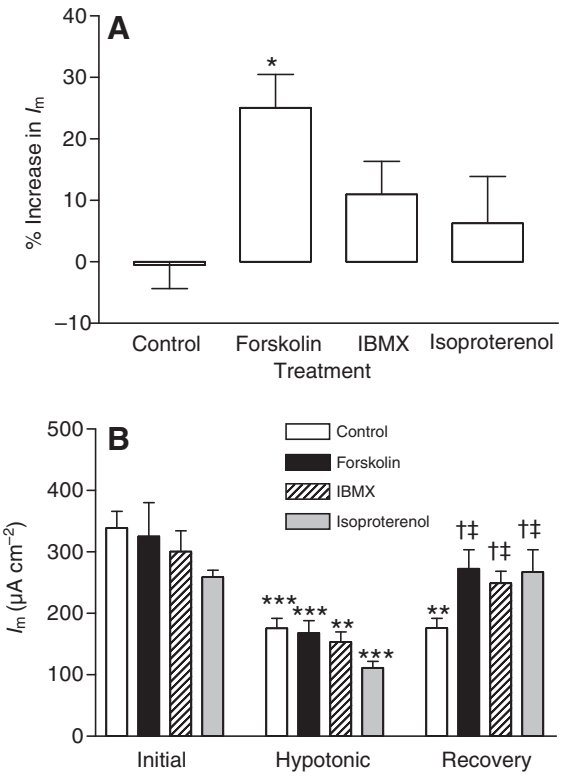

Fig. 9. Transport activation in opercular epithelia of unstimulated (isotonic) conditions and following 60 min hypotonic shock pretreatment and 10 min post-treatment with cAMP activators. (A) In isotonic epithelia, transepithelial current (I_m, μA cm^{-2}) increased with the addition of forskolin (10 μmol l^{-1}, N=6), isoproterenol (0.1 mmol l^{-1}, N=6) and IBMX (0.1 mmol l^{-1}, N=7). This increase was significant only with the addition of forskolin (*P<0.05). (B) I_m decreased significantly following hypotonic shock (**P<0.01, ***P<0.001) with no recovery in the untreated control membranes, but there was a significant increase in I_m after the addition of each cAMP activator (†P<0.01, same concentrations as in A), returning to levels not significantly different from the initial values for each group. Stimulated levels were all significantly elevated when compared with hypotonic controls run in parallel (‡P<0.05). Statistical significance was determined by paired t-tests between test and control membranes and by one way ANOVA followed by Bonferroni's post-test for differences between all groups.

by dose related inhibition of Cl^- secretion (Marshall et al., 2000). The tissue responds to hypotonic changes as small as 10 mosmol kg^{-1}, suggesting that the cells are osmosensitive. Because this occurs in isolation, is not blocked by neurotransmitter antagonists and is not mimicked by isosmotic reductions in NaCl concentration in the bathing solutions (Marshall et al., 2000), the effect is purely an osmotic sensing cellular response.

NaCl secretion is a two step process, with Cl^- entry at the basolateral membrane *via* Na^+–K^+–$2Cl^-$ cotransporter (NKCC) and apical membrane Cl^- exit *via* anion channels (CFTR). We determined that NKCC, located at the basolateral membrane, responds to hypotonic shock, apparently *via* FAK pY407. Because integrin β1 colocalizes with NKCC and as integrin is associated with mechanosensing in other systems [notably, the endothelium lining blood vessels (Lehoux et al., 2005; Hirakawa et al., 2006) (reviewed by Hynes, 2002)] we infer that integrin may be the mechanosensing element that imparts osmosensing when cell volume changes.

The present study provides strong evidence that phosphorylation of FAK at Y407 may be involved in CFTR Cl^- channel functional regulation in osmosensing MR cells of killifish opercular epithelium.

Osmosensitive phosphorylation site of FAK at Y407

Mammalian FAK protein has tyrosine phosphorylation sites at Y397, Y407, Y576, Y577, Y861 and Y925. Phosphorylated FAK at Y407, Y576/Y577 and Y861 was previously identified in zebrafish embryos (Crawford et al., 2003) and killifish opercular epithelium (Marshall et al., 2005b; Marshall et al., 2008). In addition, FAK Y576 is one of the major sites of phosphorylation by Src family kinases. Tyrosine 576 lies within the FAK catalytic domain and is part of the regulatory region of the protein. Therefore, phosphorylation at this site enhances FAK's catalytic activity (Calalb et al., 1995).

Hypotonic shock strongly inhibits Cl^- secretion and only Y407 is sensitive to hypotonic shock; dephosphorylation of FAK pY407 coincides with transport inhibition (Marshall et al., 2008). In the present study, immunocytochemistry results using phospho-antibodies to FAK pY407 and pY576 and antibody to the carboxyterminus of CFTR (–DTRL; conserved in killifish and humans) demonstrated that FAK and CFTR are colocalized in the apical crypt of MR cells of killifish opercular epithelium, thus confirming our previous finding (Marshall et al., 2008). Hypotonic treatment of opercular epithelia eliminated immunostaining of FAK pY407 in apical crypts of MR cells, but not immunostaining of FAK pY576. The latter result supports the fact that FAK has not been removed from the membrane during hypotonic treatment, but has merely been dephosphorylated uniquely at position 407. The osmosensitivity of FAK pY407 follows a similar pattern and time course to the one we observed in the basolateral membrane (Marshall et al., 2008), suggesting that FAK in basolateral and apical membranes operates similarly in the two locations. The question thus remained whether activators of the transport, recognized as agents that can increase the open channel probability of CFTR (Marshall et al., 1995; Singer et al., 1998), could rephosphorylate FAK.

In heart, FAK has been associated with volume activated, outward rectifying anion channel (VSOAC) activation and FAK/Src inhibition paradoxically enhances this Cl^- current (Walsh and Zhang, 2005), whereas a similar stimulus in the killifish dephosphorylates FAK specifically at Y407 and deactivates the CFTR anion channel. In nerves, hypotonic shock instead increases overall phosphorylation of FAK coincident with taurine efflux

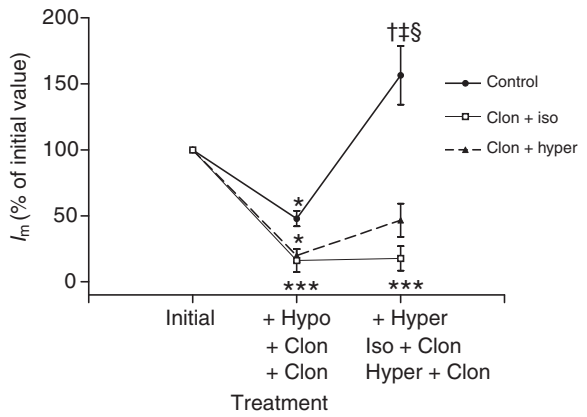

Fig. 10. Effects of hypotonicity, clonidine and hypertonicity on opercular epithelia electrophysiology. Hypotonic treatment significantly decreased I_m in resting epithelia (*P<0.05). Hypertonic shock after hypotonic inhibition significantly increased I_m (†P<0.001) to levels significantly elevated compared with the initial state (‡P<0.05, N=8). Clonidine addition (10 μmol l^{-1}, 60 min) had a more pronounced inhibitory effect than did hypotonic shock on I_m (***P<0.001) and a control isotonic rinse following clonidine incubation produced no recovery of I_m (N=18). Hypertonic shock after clonidine produced a moderate increase in I_m but this was significantly lower than without clonidine, indicating hypertonicity affecting only part of the overall clonidine inhibition. Isotonicity levels were not restored and remained significantly different from the initial state and the hypertonic treatment without drug addition (***P<0.001 and §P<0.001, N=11). Statistical significance was determined by paired t-tests between test and control membranes and by one way ANOVA followed by Bonferroni's post-test for differences between all groups.

(Lezama et al., 2005), characteristic of a regulatory volume decrease response. A closer examination of site specific FAK phosphorylation in cardiac and neural systems may reveal a common cell volume sensitive site on FAK.

Forskolin, isoproterenol and IBMX

Forskolin activates adenylate cyclase, isoproterenol is a selective β-adrenergic agonist that also increases intracellular cAMP (Marshall et al., 2000) and IBMX is a non-specific inhibitor of cAMP (and cGMP) phosphodiesterases. As a result of these pharmaceutical actions, forskolin, isoproterenol and IBMX are all known to increase cAMP levels and activate CFTR in killifish (Marshall et al., 1995; Singer et al., 1998) and mammals (reviewed by Dahan et al., 2001). We confirmed that addition of forskolin, isoproterenol and IBMX to the serosal side of opercular membrane following 80% hypotonic shock increased I_{sc} significantly. Parallel to the increase in I_{sc}, immunocytochemistry detected, after 10 min treatments of any of the three chemicals, clear rephosphorylation of FAK at Y407, compared with parallel run hypotonic controls wherein the FAK remained dephosphorylated. The rephosphorylation of FAK pY407 with hypertonicity, IBMX, isoproterenol and forskolin was marked in the apical crypt and subapical area of MR cells although FAK pY407 was present in both apical and basolateral membranes of MR cells in initial (isotonic) conditions. Thus FAK pY407 shows differential responsiveness depending on cellular location and suggests that the cell signalling pathways for CFTR and NKCC are different from each other. We interpret the fact that hypertonic solutions after clonidine could not restore I_m to control levels as a serial block of basolateral Cl⁻ entry through NKCC that was not reversed by hypertonicity and that hypertonicity may only restore apical membrane anion conductance, hence the only partial recovery to control isotonic levels.

FAK has been associated with channel activation in cell volume responses in other systems. In cardiac myocytes, FAK activation enhances the volume sensitive chloride current ($I_{Cl,swell}$), which flows through VSOAC, a DIDS sensitive anion channel, in concert with Src family kinases (Walsh and Zhang, 2005). Mammalian systems involving FAK generally go through initial autophosphorylation at Y397 followed by Src family kinase phosphorylation at Y576 and Y577. FAK phosphoryation at Y407 has been linked to both stimulatory and inhibitory actions. Gastrin releasing peptide stimulates gastric tumour cells and phosphorylates FAK at Y397 and Y407, connected to tumour cell differentiation (Matkowskyj et al., 2003), yet in NIH3T3 cells, FAK Y397 and Y407 operate inversely and FAK Y407 phosphorylation may negatively regulate these cells (Lim et al., 2007). It is clear that FAK Y407 can be phosphorylated by multiple pathways of which osmosensing is only one.

Clonidine and forskolin

To examine whether other physiological inhibitory responses paralleled the hypotonic response, we applied clonidine, the α_2-adrenoceptor agonist, which is mediated at least in part *via* inositol trisphosphate and a rise in intracellular Ca^{2+} (Marshall et al., 1993), followed by forskolin. In this case, clonidine also dephosphorylated FAK pY407 and inhibited the transport. Likewise, forskolin restored the transport and rephosphorylated FAK pY407. Thus there appears to be a convergent pathway for inhibitors and activators of the transport to FAK and CFTR, suggesting that the FAK responses are very close to those of CFTR, perhaps involving FAK phosphorylation of CFTR directly or through a nearby serine/threonine protein kinase. This also shows that FAK is not uniquely or solely affected by changes in cell volume in response to hypotonic or hypertonic shock. May and Degnan (May and Degnan, 1985), also working with *F. heteroclitus* opercular membrane, recognized early that the inhibitory and stimulatory pathways controlling NaCl secretion converged apparently on the transporters.

CFTR regulation by phosphorylation

PKA and PKC are known to activate CFTR anion channels in all systems thus far examined and the regulatory domain of CFTR has numerous (approximately 20) potential serine and threonine phosphorylation sites (reviewed by Dahan et al., 2001). These sites are well conserved from fish to mammals, while other areas of the R domain of killifish CFTR are highly divergent from the human (Singer et al., 1998). In mammals (Calu-3 airway epithelial cells), CFTR is physically and functionally associated with the serine/threonine phosphatase PP2A, which can dephosphorylate CFTR and deactivate it (Thelin et al., 2005). Of interest here are two potential tyrosine phosphorylation sites in the R domain at positions Y808 and Y849/Y852 that are well conserved from teleost to human. These tyrosines provide potential sites for direct tyrosine phosphorylation of CFTR by FAK or other tyrosine kinases. Feasibly, tyrosine phosphorylation of CFTR might provide more sustained activation, not subject to immediate dephosphorylation by PP2A. As a general characteristic in patch clamp studies, CFTR channels 'run down' quickly unless continuously activated by PKA; in turn, the rundown is possibly through the action of PP2A. As well, there remains the possibility that FAK could activate other kinases that would activate CFTR by serine/threonine phosphorylation in a more conventional manner. The tyrosine kinase inhibitor genistein has been shown to stimulate Cl⁻ secretion in (normal quiescent) shark rectal gland (Lehrich and Forrest, 1995) and killifish opercular membrane [if previously inhibited (Marshall et al., 2000)] and to inhibit Cl⁻ secretion in previously stimulated

opercular membrane (Marshall et al., 2000), possible indicators of tyrosine regulation of NaCl secretion. Genistein's role is recognized to be complex (both stimulatory and inhibitory) and dose dependent, and genistein had effects on CFTR channels directly, not mediated through activation state (Wang et al., 1998). At least in the killifish, the kinase inactive analogue daidzein has no effect (Marshall et al., 2000; Marshall et al., 2005b), suggesting a real tyrosine kinase based regulation of transport. Now that the tyrosine kinase has been identified (FAK) more specific inhibitors will be valuable in connecting FAK to potential targets.

CFTR in human and killifish possesses a PDZ binding domain in the carboxyterminus (Singer et al., 1998) and *via* this domain appears to interact with the regulatory protein Na^+/H^+ exchanger regulatory factor (NHERF) (Naren et al., 2003; Li et al., 2005), which through a FERM binding domain at the carboxyterminus in turn interacts with FERM *via* a domain on ezrin. Thus the concept of a regulatory complex involving FERM binding domains pre-exists with an apparent stoichiometry of 2 CFTR:1 NHERF:1 ezrin (Li et al., 2005). As with NHERF, FAK also possesses the FERM binding domain at the aminoterminus (Parsons, 2003) and thus may form an alternative complex with CFTR and ezrin. This is reasonable, as seawater apical membranes of MR cells are not known to contain the Na^+/H^+ exchanger, nor presumably the NHERF, as this is a freshwater type conformation in some species for freshwater adaptive ion uptake. Because rephosphorylation of FAK pY407 is a common feature of cAMP dependent (forskolin, isoproterenol, IBMX) and cAMP independent hyperosmotic stimulation of CFTR mediated Cl^- secretion, it is reasonable to believe that the two pathways converge on FAK, thus implying a close relationship between FAK and CFTR.

CFTR and apical membrane turnover

The rapid (about 1 h) onset of Cl^- secretion in *Fundulus* entering seawater, i.e. before *de novo* protein expression could contribute, involves the movement of pre-existing CFTR from subapical vesicles into the apical membrane (Marshall et al., 2005b; Shaw et al., 2008). Shaw and colleagues (Shaw et al., 2008) measured CFTR surface appearance using cell surface biotinylation, while Marshall and colleagues (Marshall et al., 2005b) observed CFTR disappearance from the subapical locus by immunocytochemistry. Investigations of a potential role for serum and glucocorticoid inducible kinase-1 (SGK1) have revealed that SGK1 augments human CFTR Cl^- currents in *Xenopus* oocytes (Sato et al., 2007). Further, rapid transfer to seawater increases SGK1 mRNA expression followed quickly by an increase in CFTR based Cl^- secretion by killifish opercular membranes. Apparently the SGK1 effect is not *via* the cortisol glucocorticoid receptors, in spite of the fact that the glucocorticoid receptor is the steroid receptor type associated with successful seawater adaptation in killifish (Marshall et al., 2005a; Shaw et al., 2007).

The finding of CFTR and FAK pY407 by immunogold TEM in subapical vesicles is consistent with the redistribution of CFTR we have observed previously (Marshall et al., 2002), wherein CFTR protein moves from assembly areas near the nucleus to a subapical position and then into the apical membrane. This pattern follows the CFTR turnover concept where the subapical vesicles provide a reserve of transport protein that can be inserted into the apical membrane to increase transport capacity. The vesicles could also represent recycled apical membrane removed from the apical membrane in this dynamic exchange. In mammalian systems CFTR has a half-life of about 24 h, implying a continuous turnover of CFTR to and from the apical membrane (Swiatecka-Urban et al., 2002). CFTR mRNA expression in seawater is elevated relative to that in freshwater in killifish (Marshall et al., 1999; Scott et al., 2008) and in Japanese eel (Tse et al., 2006) but not in striped bass (Madsen et al., 2007). Whereas some euryhaline teleosts have inducible augmented CFTR expression, the situation in bass suggests that CFTR expression is equal in the two salinities, i.e. bass are always ready for seawater exposure (Madsen et al., 2007). In either case, CFTR (and presumably also FAK) proteins are likely to play a role in continual turnover involving cycling of subapical membrane vesicles. Also of importance, there is a tyrosine in the carboxyterminus (Y1424) that serves along with I1427 as an internalization cue for endocytosis *via* clathrin coated pits (Peter et al., 2002). The possibility that tyrosine phosphorylation could regulate the activation and turnover of CFTR opens another possible role for FAK in CFTR containing epithelia.

LIST OF ABBREVIATIONS

cAMP	cyclic AMP
CFTR	cystic fibrosis transmembrane conductance regulator
FAK	focal adhesion kinase
FERM	protein 4.1, ezrin–radixin–moesin
$I_{Cl,swell}$	volume sensitive chloride current
I_m	transepithelial current
I_{sc}	short circuit current
MR cells	mitochondria rich cells
PKA	protein kinase A
PKC	protein kinase C
PP2A	protein phosphatase 2A
R domain	regulatory domain
R_t	transepithelial resistance
SGK1	serum and glucocorticoid inducible kinase-1
TEM	transmission electron microscopy
V_t	transepithelial potential
VSOAC	volume sensitive outward rectifying anion channel

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