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RESEARCH ARTICLE

Nitric oxide inhibition of NaCl secretion in the opercular epithelium of seawater-acclimated killifish, *Fundulus heteroclitus*

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ABSTRACT

Nitric oxide (NO) modulates epithelial ion transport pathways in mammals, but this remains largely unexamined in fish. We explored the involvement of NO in controlling NaCl secretion by the opercular epithelium of seawater killifish using an Ussing chamber approach. Pharmacological agents were used to explore the mechanism(s) triggering NO action. A modified Biotin-switch technique was used to investigate *S*-nitrosation of proteins. Stimulation of endogenous NO production via the nitric oxide synthase (NOS) substrate L-arginine (2.0 mmol l^{-1}), and addition of exogenous NO via the NO donor SNAP (10^{-6} to 10^{-4} mol l^{-1}), decreased the epithelial short-circuit current (I_{sc}). Inhibition of endogenous NO production by the NOS inhibitor L-NAME (10^{-4} mol l^{-1}) increased I_{sc} and revealed a tonic control of ion transport by NO in unstimulated opercular epithelia. The NO scavenger PTIO (10^{-5} mol l^{-1}) supressed the NO-mediated decrease in I_{sc}, and confirmed that the effect observed was elicited by release of NO. The effect of SNAP on I_{sc} was abolished by inhibitors of the soluble guanylyl cyclase (sGC), ODQ (10^{-6} mol l^{-1}) and Methylene Blue (10^{-4} mol l^{-1}), revealing NO signalling via the sGC/cGMP pathway. Incubation of opercular epithelium and gill tissues with SNAP (10^{-4} mol l^{-1}) led to *S*-nitrosation of proteins, including Na^+/K^+-ATPase. Blocking of NOS with L-NAME (10^{-6} mol l^{-1}) or scavenging of NO with PTIO during hypotonic shock suggested an involvement of NO in the hypotonic-mediated decrease in I_{sc}. Yohimbine (10^{-4} mol l^{-1}), an inhibitor of α_2-adrenoceptors, did not block NO effects, suggesting that NO is not involved in the α-adrenergic control of NaCl secretion.

KEY WORDS: NO, Cl^- secretion, Mummichog, Epithelial ion transport, sGC/cGMP pathway

INTRODUCTION

The gasotransmitter nitric oxide (NO) is known to mediate various physiological functions in vertebrates by acting as an autocrine and paracrine signalling molecule. NO is produced during the conversion of L-arginine to L-citrulline by NO synthase (NOS). It typically exerts its physiological functions by binding directly to the ferrous iron of haemproteins [like the soluble guanylyl cyclase (sGC), its most sensitive target] or indirectly by *S*-nitrosation of protein thiol groups (-SH groups) (Thomas et al., 2008; Hill et al., 2010). In fish, two isoforms of NOS have been identified: a constitutive, Ca^{2+}/calmodulin-dependent isoform NOS1 (also called neuronal or nNOS) and an inducible, Ca^{2+}/calmodulin-independent isoform NOS2 (also called inducible or iNOS). A paralogue of NOS3 (also called endothelial or eNOS) has not been identified in fish genomes (Øyan et al., 2000; Andreakis et al., 2011).

Emerging studies suggest that NO can modulate epithelial ion transport pathways in fish (Evans, 2002; Evans et al., 2004; Trischitta et al., 2007; Perry et al., 2016) but information is limited compared with that for mammals. The gill epithelium is the primary site of osmoregulation in fishes and expresses many of the ion transport proteins found in the mammalian nephron. In certain teleost fish species, the opercular epithelium is an accepted two-dimensional surrogate model system for investigation of ion secretion in the branchial epithelium, as the two tissues express the same cell types. Electrophysiology studies investigating NaCl transport functions of teleost fish gill are numerous (see review by Marshall and Bellamy, 2010), notably on seawater fish. The euryhaline killifish *Fundulus heteroclitus* has proved to be an excellent model system for the study of ion secretion processes and their endocrine and neural control (Karnaky et al., 1976; Foskett and Scheffey, 1982; Marshall et al., 1997; Marshall and Bellamy, 2010). The NaCl secretion mechanism by marine teleost fish gill is therefore well established. Briefly, seawater-acclimated killifish actively secrete chloride (Cl^-) through a transcellular pathway involving basolateral $Na^+/K^+/2Cl^-$ (NKCC) cotransporters and Na^+/K^+-ATPase (NKA) in series with apical cystic fibrosis transmembrane conductance regulator (CFTR) anion channels (Wood and Marshall, 1994; Marshall and Bryson, 1998; Marshall et al., 2000). This allows measurement of the net active transport of Cl^- across the epithelium while Na^+ diffuses through a paracellular pathway (Karnaky et al., 1976; Degnan et al., 1977). A regulatory role of NO in ion transport was suggested by the observed down-regulation of NKA activity by NO donors in the kidney and gill of freshwater-acclimated brown trout and in the gill of seawater-acclimated Atlantic salmon (Tipsmark and Madsen, 2003; Ebbesson et al., 2005). Demonstration of NOS expression in osmoregulatory tissues (gill, opercular epithelium, kidney and intestine) in a variety of fish species (Mauceri et al., 1999; Evans, 2002; Ebbesson et al., 2005; Hyndman et al., 2006; Pederzoli et al., 2007; Porteus et al., 2015) also suggested an involvement of NO in ion regulation. To our knowledge, only two studies have demonstrated the effect of exogenous NO production on NaCl transport in fish (Evans et al., 2004; Trischitta et al., 2007). However, the mechanism(s) of NO action on ion transport in fish remains unclear. Once generated, NO can act as a potent signalling molecule and influence various physiological functions via paracrine or neuroendocrine routes. The sGC, which drives cyclic guanosine monophosphate (cGMP) production, is a key mediator of NO-dependent effects and the most sensitive target of NO, making the cGMP-mediated signalling pathway a good candidate for the NO effect on ion transport. NO can also exert its biological effects

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List of abbreviations

CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
I_{sc}	epithelial short-circuit current
L-NAME	N_{ω}-nitro-L-arginine methyl ester
MB	Methylene Blue
NEM	*N*-ethylmaleimide
NKA	Na^+/K^+-ATPase
NKCC	$Na^+/K^+/2Cl^-$ cotransporter
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	*H*-(1,2,4)oxadiazole(4,3-a)quinoxaline-1-one
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
R_t	transepithelial resistance
sGC	soluble guanylyl cyclase
-SH	protein thiol groups
SNAP	*S*-nitroso-*N*-acetyl-DL-penicillamine
SNP	sodium nitroprusside
SPNO	spermine NONOate
V_t	transepithelial potential

indirectly through formation of nitrosating agents (e.g. N_2O_3) that *S*-nitrosate proteins, including ion transport proteins, thereby affecting their activity (Hill et al., 2010). Studies showed that α-subunits of NKA can be *S*-nitrosated after exogenous NO administration in mammals (Sato et al., 1995; Jaffrey et al., 2001; Althaus et al., 2011), making it a possible mechanism for NO actions on ion transport also in fish. Yet, only one study in the heart of eel (*Anguilla anguilla*) has demonstrated a modulatory role of *S*-nitrosation of the protein phospholamban in the Frank–Starling response (Garofalo et al., 2009). Evans et al. (2004) first demonstrated the capacity of several paracrine agents, including NO, to inhibit epithelial ion transport in fish, and suggested a signalling pathway for its actions that included stimulation of NOS activity by endothelin; but the downstream mechanism for NO action could not be clearly identified. A less well investigated pathway for NO is via the sympathetic nervous system. Marshall et al. (1998) showed that seawater-acclimated killifish can decrease their Cl^- secretion rate through the action of the sympathetic nervous system mediated by α_2-adrenoceptors – a Ca^{2+}-dependent pathway. This offers a potential route for NO effects, notably for the calcium-dependent and neuronal isoform (NOS1). Hence, the goal of the present study was (1) to investigate the regulatory role of NO donors and NOS enzyme inhibitors and promoters on ion transport across the opercular epithelium of seawater-acclimated killifish and (2) to uncover the underlying mechanism(s) of transport regulation by NO by evaluating various molecular mechanisms known or suspected to control NaCl transport in fish. We investigated the direct binding of NO to haem in sGC with further signal transduction via the formation of cGMP, and the indirect *S*-nitrosation of protein -SH groups, proceeding via intermediate formation of nitrosating agents (e.g. N_2O_3). We also clarified whether NO is involved in other established pathways affecting Cl^- secretion, viz. the α-adrenergic- and hypotonic-mediated decrease of Cl^- secretion (Marshall et al., 1998, 2000).

MATERIALS AND METHODS

Animals

All experiments were carried out on adult killifish, *Fundulus heteroclitus* (Linnaeus 1766) (4–12 g, *N*=59) of both sexes. Fish were obtained from the Antigonish estuary (Antigonish, NS, Canada) in spring 2015 and maintained in indoor holding facilities containing seawater (32 ppt) at 24°C and ambient photoperiod under artificial light. Fish were fed with marine fish food blend (Nutrafin flakes and tubifex worms; R.C. Hagen, Montreal, QC, Canada) twice daily. Fish were individually netted and killed by spinal cord sectioning and pithing. Paired opercular epithelia (~1 cm^2) were immediately dissected from the left and right underlying opercular bone under a microscope while kept moist with 305 mOsm kg^{-1} Cortland's saline (composition in mmol l^{-1}: NaCl 160, KCl 2.6, $CaCl_2$ 1.6, $MgSO_4$ 0.9, $NaHCO_3$ 17.9, NaH_2PO_4 3.0 and glucose 5.6).

Experimental set-up and protocol

Electrophysiology of ion transport

Dissected paired opercular epithelia were pinned out flat over the circular aperture of the Ussing chamber insert, giving an exposed epithelium area of 0.125 cm^2, and mounted vertically in the Ussing chamber. The two 4.0 ml hemi-chambers (serosal and mucosal baths) of the Ussing chamber set-up were filled with Cortland's saline. The Cortland's saline solution was equilibrated with 99% O_2/1% CO_2 and pH was 7.8. Any adherent mucus was rinsed away and the rim area was lightly greased to avoid solution leaking and to minimize edge damage. Chambers were surrounded by a water jacket controlled at 26±1°C and were stirred vigorously by magnetic fleas. The time between the start of dissection and the completion of mounting was ~15 min. Hypotonic shock, used in some experiments, was applied by flushing of the hemi-chambers with diluted Cortland's saline (80 ml Cortland's saline+20 ml deionized water, 244 mOsm kg^{-1}). Symmetrical solutions were always applied to avoid effects of asymmetrical solutions, and the chambers were filled at an equal rate to avoid hydrostatic pressure across the epithelium.

For each experiment, paired epithelia from one animal were mounted in two separate Ussing chamber set-ups, with one serving as a parallel running control, allowing pairwise measurement of electrophysiological variables. Two transmembrane electrophysiological variables were monitored with a current–voltage clamp: the transepithelial potential V_t (in mV) and the transepithelial resistance R_t (in Ω cm^2). Epithelia were kept under open circuit conditions and R_t was calculated from the deflection in V_t generated by injection of a 10 µA current pulse every minute. The variables were continuously recorded on LabScribe v3.0 (iWork, Dover, NH, USA). The short-circuit current, I_{sc} (µA cm^{-2}), was determined using the recorded V_t and R_t.

Seawater-acclimated killifish opercular epithelia developed a steady state over ~45 min, which was defined as the 'initial' state of the epithelium throughout the study, with an initial I_{sc} of 240±9.5 µA cm^{-2}, a R_t of 83±2.8 Ω cm^2 and a V_t of 20.1±0.5 mV (*N*=54). When epithelia had stabilized and reached their initial steady state, drug was added (or solution changed, if needed), and the pair-wise recordings were continued until a new steady-state I_{sc} was reached in response to treatments (not shown in figures).

Chemicals and their experimental application

All the drugs were obtained from Sigma-Aldrich (St Louis, MO, USA). L-Arginine (2.0 mmol l^{-1}) was used to stimulate the endogenous production of NO by NOS. The NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; at 10^{-4}, 10^{-5} and 10^{-6} mol l^{-1}) was used to release NO in the medium and investigate the effect of exogenous NO. N_{ω}-nitro-L-arginine methyl ester (L-NAME) (10^{-4} and 10^{-5} mol l^{-1}), a NOS inhibitor, was used to inhibit endogenous NO production. The NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; 10^{-5} mol l^{-1}) was used to block baseline NO production by the tissue. The guanylate

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cyclase inhibitors Methylene Blue (MB; 10^{-4} mol l^{-1}) and *H*-(1,2,4) oxadiazole(4,3-a)quinoxaline-1-one (ODQ; 10^{-6} mol l^{-1}) were used to evaluate the participation of cGMP-dependent pathways. *N*-Ethylmaleimide (NEM; 10^{-4} mol l^{-1}), a reagent that forms a strong C–S bond to free -SH groups of proteins, was used to 'simulate' the covalent modification of -SH groups that also occurs by *S*-nitrosation. Clonidine, an α_2-adrenoceptor agonist (10^{-9} to 10^{-6} mol l^{-1}) was used to confirm the use of yohimbine as an α_2-adrenoceptor blocker in fish. Yohimbine (10^{-4} mol l^{-1}) was then used to investigate whether NO interacts with the adrenergic pathway. Isoproterenol (10^{-4} mol l^{-1}), a β-adrenoceptor agonist, was used to stimulate the I_{sc} after incubation with SNAP and confirm the responsiveness of the epithelium to other stimuli.

Stock solutions of ODQ and SNAP were dissolved in dimethyl sulphoxide (DMSO). SNAP was first dissolved in DMSO but made up to the desired concentration by further dilution in Cortland's saline. Yohimbine, PTIO and NEM were dissolved in 70% ethanol. L-NAME, L-arginine, clonidine, isoproterenol and MB were dissolved in Cortland's saline. Drugs were prepared to 1% dose accuracy (e.g. 10^{-4} mol l^{-1} means 1.00×10^{-4} mol l^{-1}). The various solutions were stored either frozen (−20°C) or at 4°C as required.

In all experiments, drugs were added to the serosal side of the opercular epithelium, while the solvent was added to a parallel-run paired control epithelium from the same animal. Equal volumes of solution (drug versus solvent) were also added to both sides of the tissues to avoid volume and osmotic effects. The final volume of drug/solvent added ranged from 0.1% to 1% of the initial volume of the experimental medium. The maximum amount of DMSO added with a drug was 0.1% of chamber volume, a level without noticeable effect on the I_{sc}.

Generally, drugs were allowed 15 min to get to their target, and significant changes (if any) were visible on the raw recording accordingly.

Biotin-switch assay for the determination of *S*-nitrosated proteins

In order to identify *S*-nitrosated proteins, a modified biotin-switch method (Jaffrey and Snyder, 2001) was used (*S*-Nitrosylated Protein Detection Assay Kit, item no. 10006518, Cayman Chemical, Ann Arbor, MI, USA). The method consists of three steps: (1) blocking of the free, non-nitrosated -SH groups with the blocking agent NEM, (2) cleavage of any S–NO bonds present by sodium ascorbate, and (3) biotinylation of the newly formed SH groups using 3-(*N*-maleimido propionyl) biocytin, facilitating the detection of *S*-nitrosated proteins by western blotting. Both gill (5.6±0.50 mg) and opercular epithelium (2.4±0.26 mg) tissues were dissected out from five fish (both sexes, ∼11.9±2.3 g) and placed in ice-cold Cortland's saline equilibrated with 99% O_2/1% CO_2 with occasional shaking until completion of the dissections. Tissues were then transferred into 24-well cell culture plates (Corning Costar 3524, Sigma-Aldrich) containing 1 ml of ice-cold Cortland's saline equilibrated with 99% O_2/1% CO_2. The right-side gills and opercular epithelia were incubated with SNAP (10^{-4} mol l^{-1}) and the left-side gills and opercular epithelia were used as a paired control. A concentrated stock solution of SNAP was freshly made before the incubation. The final concentration of SNAP was obtained by dilution of the SNAP stock solution in the 1 ml of Cortland's saline already added to each well. Then the plates were immediately transferred to a bag gassed with 99% O_2/1% CO_2 and incubated in an incubator-shaker (model 1570, VWR, Montreal, QC, Canada) at 26°C and 170 rpm for 70 min (10 min for warming up and 60 min regular incubation). Tissues were transferred to P-SNO Cell Lysis Buffer (Buffer A, item no. 10006520, Cayman Chemical) and kept at −80°C until analysis. Tissues were homogenized in Buffer A containing 1:100 protease inhibitor cocktail (P8340, Sigma-Aldrich) and centrifuged 10 min at 1000 ***g*** to eliminate major debris. Blocking of free -SH groups, reduction of *S*-NO groups, and biotinylation of the protein samples were performed according to the manufacturer's instructions. Control samples were duplicated: one set was treated identically to the SNAP-incubated samples to reveal endogenously *S*-nitrosated proteins; in the other set, the reducing and biotin-labelling steps of the assay were omitted to observe endogenously biotinylated proteins. Samples were kept at −20°C until analysis by western blotting. A total amount of 50 µg of total proteins was loaded and separated on a 12% polyacrylamide gel using NuPAGE® MES SDS running buffer (Invitrogen, Waltham, MA, USA) at 200 V for 35 min. Proteins were then transferred to a nitrocellulose membrane (Amersham™ Protran™ 0.45 µm NC, GE Healthcare, Brondby, Denmark) using a Tris-glycine transfer buffer (7.5 mmol l^{-1} Tris, 60 mmol l^{-1} glycine, 20% v/v methanol) for 2 h at 25 V. Blots were washed 4×5 min with Buffer B (item no. 10006521, Cayman Chemical) containing 0.1% Triton X-100 before incubation with detection reagent II (1:75, fluorescein, item no. 10006525, Cayman Chemical) in Buffer B containing 0.1% Triton X-100 for 1 h at room temperature (RT). After 4×5 min washing steps with Buffer B containing 0.1% Triton X-100, blots were air-dried and scanned with a Typhoon™ FLA 9500 laser scanner (GE Healthcare), using a laser line of ∼490 nm excitation and ∼525 nm emission for detection of *S*-nitrosated proteins. Blots were then re-probed with a mouse monoclonal anti-chicken NKA antibody (1:500, α5 antibody; developed by Douglas M. Fambrough and obtained from the Developmental Studies Hybridoma Bank created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA, USA) in Tris-buffered saline containing Tween 20 (TBS-T; 20 mmol l^{-1} Tris, 140 mmol l^{-1} NaCl, 1% Tween 20) for 1 h at RT. Blots were washed 4×5 min before incubation with the secondary antibody in TBS-T (1:3000, goat anti-mouse IgG, Cy3®, Invitrogen, Waltham, MA, USA) for 30 min at RT. Blots were then washed 4×5 min in TBS-T buffer and subsequently air-dried and scanned using a laser line of ∼550 nm excitation and ∼570 nm emission for detection of NKA proteins. Estimated molecular mass and merged pictures of the *S*-nitrosated protein and NKA bands suggested the presence of *S*-nitrosated NKA proteins after incubation with SNAP. To further confirm the presence of *S*-nitrosated NKA, a streptavidin pull-down assay was performed following the manufacturer's instructions. This permitted purification/isolation of *S*-nitrosated proteins (i.e. biotinylated proteins) and confirmation of the presence of *S*-nitrosated NKA among the *S*-nitrosated proteins detected earlier using the anti-NKA antibody and methods described above (gel electrophoresis and western blot). Briefly, 20 µg of total protein was used and brought to a final volume of 300 µl with TBS-T and incubated with 50 µl washed streptavidin beads (Pierce™ Streptavidin Magnetic Beads) overnight at 4°C with gentle mixing. The supernatant (containing non-*S*-nitrosated proteins) was removed from the beads by aspiration. The beads were washed three times with TBS-T prior to elution of *S*-nitrosated proteins with NuPAGE® LDS sample buffer (X1) and heating at 96°C for 5 min. ImageJ gel analyser (v1.50i, National Institutes of Health; Schneider et al., 2012) was used for the relative densitometric analysis of *S*-nitrosated NKA.

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Statistical analyses

The data are presented as means±s.e.m. and I_{sc} is reported as percentage of the initial I_{sc}. Percentage datasets were arcsine transformed to meet the statistical assumption of homogeneity of variance prior to repeated measures two-way ANOVA. Paired two-tailed Student's tests were performed on the densitometric data. *N* indicates the number of fish used. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) and $P<0.05$ was regarded as significant.

RESULTS

Effect of endogenous NO on ion transport

Incubation with 2.0 mmol l^{-1} L-arginine, used to stimulate NOS-catalysed NO generation, induced a 48.6±2.9% (*N*=3) decrease of I_{sc} (Fig. 1).

The NOS inhibitor L-NAME was used to determine the contribution of endogenous NO to ion transport in unstimulated tissues. Pilot experiments using symmetrical isotonic Cortland's solution (not shown) showed that 15 min incubation with 10^{-6} mol l^{-1} L-NAME had no (stimulatory) effect on I_{sc}. Therefore, incubation time was increased and hypotonic solutions were applied to decrease the initial steady-state I_{sc} to better explore the effect of L-NAME (Fig. 2A). Hypotonic shocks induced a 74.9±2.9% decrease in I_{sc} within 20 min on average in the controls of the three separate experiments conducted (Fig. 2). Incubation with 10^{-6} mol l^{-1} L-NAME (Fig. 2A) reduced by 24.7±2.4% (*N*=5) the hypotonic shock-induced decrease in I_{sc}, suggesting a potential contribution of NO to the 'shut down' of Cl^- secretion when fish face a marked drop in salinity. To confirm this, two complementary experiments were conducted: (1) epithelia were incubated with L-NAME at a higher concentration (10^{-4} mol l^{-1}) to block NOS activity or (2) epithelia were incubated with PTIO (10^{-5} mol l^{-1}) to scavenge NO without affecting NOS. In both cases, drugs were added before and after hypotonic shock to ensure their presence during the full experiment. Pre-incubation with L-NAME at 10^{-4} mol l^{-1} induced a 14.3±1.2% (*N*=5) increase in the initial I_{sc} (Fig. 2B), revealing a tonic control of ion transport by NO; however, there was no effect on the hypotonic shock-induced decrease in I_{sc}. Epithelia incubated with PTIO showed a reduced hypotonic I_{sc} decrease (by 59.6±0.4%, *N*=3, of the initial I_{sc}) compared with the controls (by 69.4±2.7%, *N*=3, of the initial I_{sc}) (Fig. 2C), suggesting the involvement of NO in the hypotonic-mediated decrease. Pre-incubation with PTIO had no effect on the initial I_{sc} (Fig. 2C).

Effect of exogenous NO on ion transport

We tried to mimic the effect of endogenous NO on epithelial ion transport by using the NO donor SNAP at different concentrations (10^{-6}, 10^{-5} and 10^{-4} mol l^{-1}). SNAP at 10^{-6}, 10^{-5} and 10^{-4} mol l^{-1} induced a 44.6±6.9% (*N*=8), 37.0±2.6% (*N*=5) and

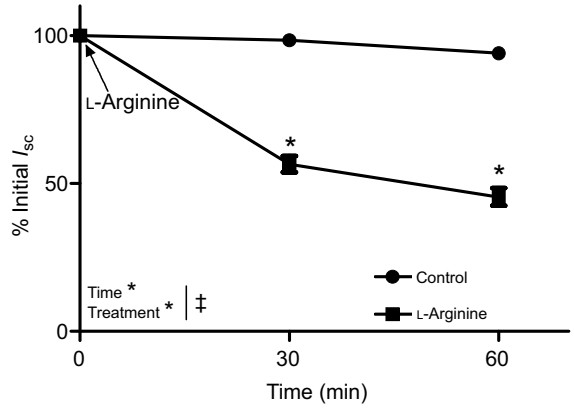

Fig. 1. Effect of L-arginine on the epithelial short-circuit current, I_{sc}. For each experiment, paired epithelia were used with one serving as a control and the second incubated with 2 mmol l^{-1} L-arginine for 60 min. Data are shown as a percentage of the initial I_{sc}. Values are means±s.e.m. (*N*=3). The overall statistical influence of each factor (treatment and time) is indicated in the figures by an asterisk (bottom left) while their interaction, if any, is indicated by a double dagger (‡). The effect of treatment over time is further specified at each time point by an asterisk (Bonferroni's multiple comparison tests).

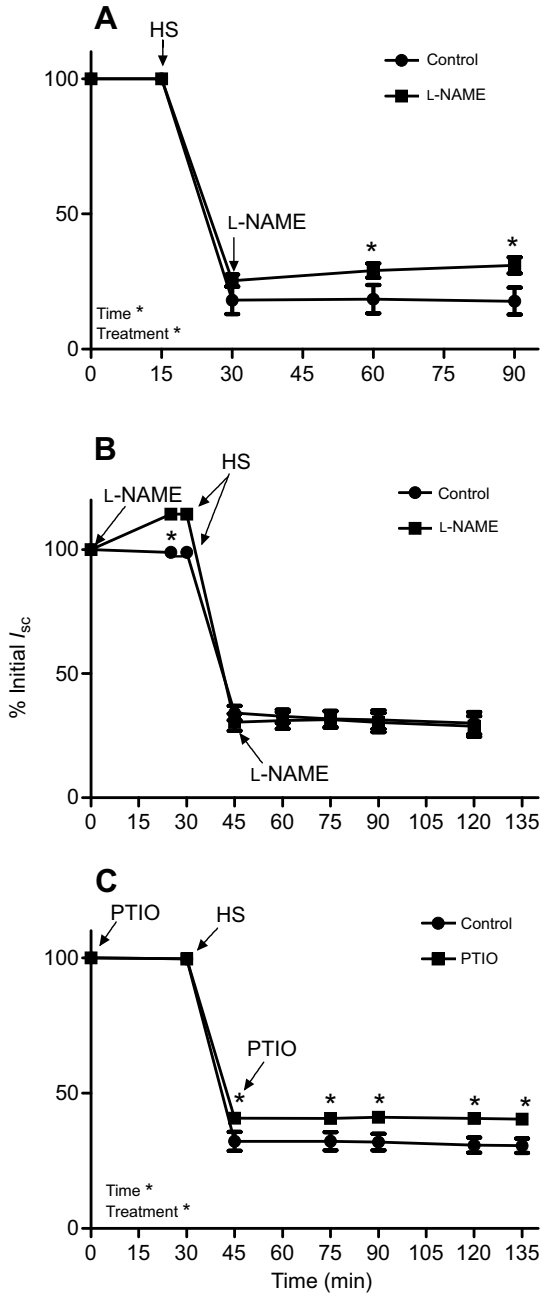

Fig. 2. Effect of hypotonic shock on I_{sc} and its modulation by the NOS inhibitor L-NAME and the NO scavenger PTIO. (A) Incubation with L-NAME (10^{-6} mol l^{-1}; *N*=5) after a hypotonic shock (HS). (B) Incubation with L-NAME (10^{-4} mol l^{-1}; *N*=5) before and after a hypotonic shock. (C) Incubation with PTIO (10^{-5} mol l^{-1}; *N*=3) before and after a hypotonic shock. Other details are as in Fig. 1.

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44.6±5.6% (N=5) decrease on the initial I_{sc}, respectively (Fig. 3), which was similar to the decrease evoked by stimulating endogenous NO generation by L-arginine (Fig. 1). The SNAP-mediated decrease was time dependent. After 30 min of incubation, all three concentrations produced a similar I_{sc} decrease; however, maximal inhibition and new steady states were reached after 60 min at the highest concentration (10^{-4} mol l^{-1}) and after 90 min at the two lower concentrations (10^{-5} and 10^{-6} mol l^{-1}) of SNAP (Fig. 3). To confirm that the observed effect was due to release of NO, we tested SNAP in the presence and absence of the NO scavenger PTIO. Pre-incubation with 10^{-5} mol l^{-1} PTIO abolished the SNAP-mediated I_{sc} decrease (Fig. 4); this pre-incubation with PTIO did not affect initial I_{sc} (Fig. 4).

Signalling mechanisms

Three potential mechanisms by which NO may regulate ion transport were investigated: (1) the sGC-cGMP pathway, (2) *S*-nitrosation of proteins and (3) interference with adrenergic pathways.

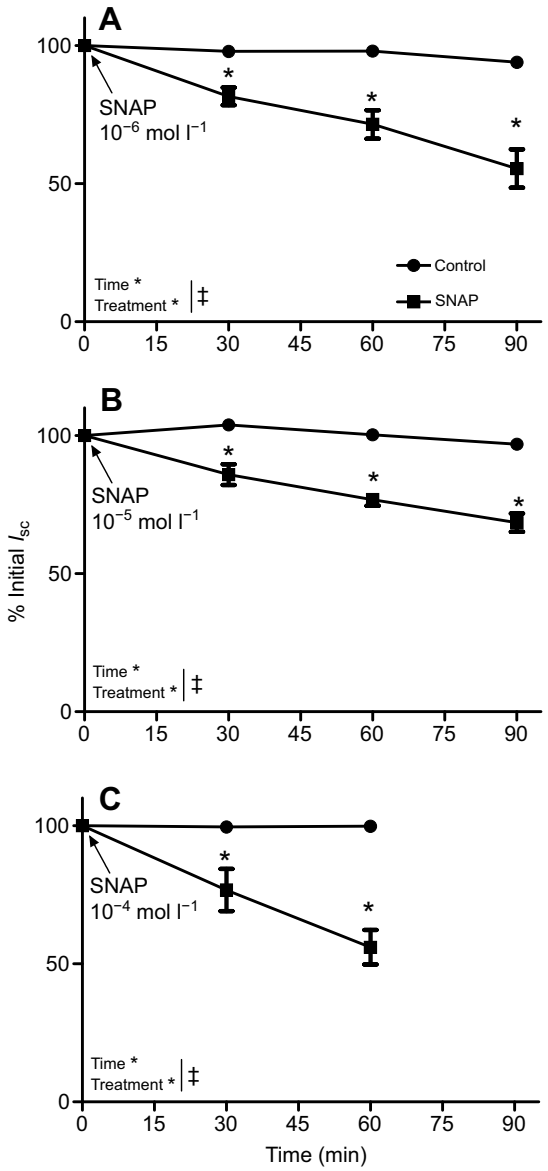

Fig. 3. Effect of three different concentrations of the NO donor SNAP on I_{sc}. Incubation with SNAP at (A) 10^{-6} mol l^{-1} (N=8), (B) 10^{-5} mol l^{-1} (N=5) and (C) 10^{-4} mol l^{-1} (N=5). Other details are as in Fig. 1.

sGC/cGMP dependence

The most sensitive target of NO is sGC. To evaluate whether sGC activation and cGMP formation are involved in NO-induced I_{sc} changes, we applied two different sGC inhibitors: MB and ODQ. Pre-incubation with MB (10^{-4} mol l^{-1}) and ODQ (10^{-6} mol l^{-1}) blocked 89.8±5.9% (N=6) (Fig. 5A) and 83.7±1.9% (N=6) (Fig. 5B) of the NO-induced I_{sc} decrease produced by the highest concentration of SNAP (10^{-4} mol l^{-1}) used, respectively. MB (10^{-4} mol l^{-1}) alone did not affect the initial I_{sc} while ODQ (10^{-6} mol l^{-1}) alone slightly but significantly decreased the initial I_{sc} in the control group (6.3±1.3%; N=6).

S-Nitrosation of proteins

A mechanism by which NO regulates the activity of proteins is through the formation of nitrosating agents, producing *S*-nitrosated proteins [formally, the addition of nitrosonium (NO^+) to -SH groups of cysteine residues]. The first approach was indirect and consisted of comparing the effect of NO with that of NEM. NEM reacts with free -SH groups of protein cysteines (forming a strong C–S bond), and thus blocks the same groups that can potentially be nitrosated by NO products. Addition of NEM alone significantly decreased the initial I_{sc} by 50.7±9.4% (N=5) within 30 min (Fig. 6), which was similar to but more rapid than the SNAP-mediated decrease (i.e. 60 min) (Fig. 6), suggesting an inhibition of the I_{sc} by modification of -SH groups. Addition of NEM after NO release by SNAP (10^{-4} mol l^{-1}) had no additional effect (Fig. 6).

In the second approach, we directly investigated *S*-nitrosation of proteins, using a modified biotin-switch technique combined with western blotting. Numerous protein bands were *S*-nitrosated after treatment with the NO donor SNAP at 10^{-4} mol l^{-1} in both opercular epithelium (Fig. 7A) and gill (Fig. 8A), apparently with a similar molecular mass pattern on the two blots. Endogenous *S*-nitrosated and biotinylated proteins were below the detection limit (Figs 7A and 8A). Interestingly, in both tissues, an *S*-nitrosated band with a molecular mass similar to that of the NKA transporter was detected. Subsequent immunoblotting of the same blots with an anti-NKA antibody confirmed the co-localization of the NKA band with that of the *S*-nitrosated band (Figs 7A and 8A), suggesting

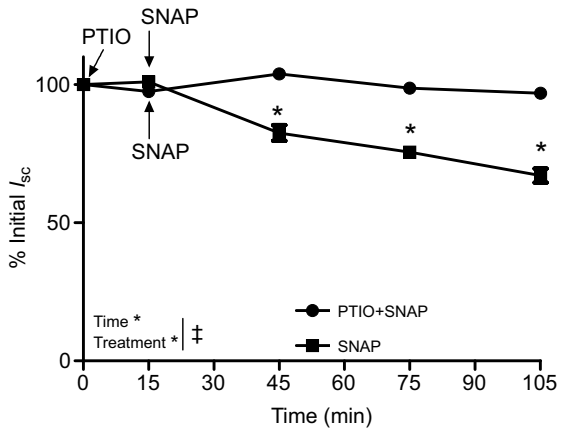

Fig. 4. Effect of the NO donor SNAP on I_{sc} with or without pre-incubation with the NO scavenger PTIO. Preincubations were carried out with 10^{-5} mol l^{-1} PTIO, followed by incubation with 10^{-5} mol l^{-1} SNAP (N=5). Other details are as in Fig. 1.

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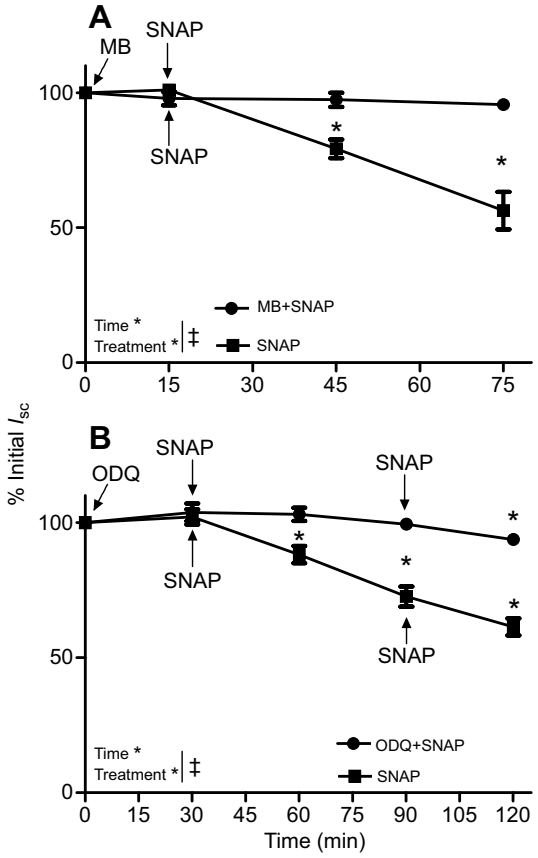

Fig. 5. Effect of the NO donor SNAP on I_{sc} with or without pre-incubation with two different sGC inhibitors: Methylene Blue (MB) and ODQ. Preincubations were carried out with (A) MB (10^{-4} mol l^{-1}; *N*=6), followed by incubation with 10^{-4} mol l^{-1} SNAP or (B) ODQ (10^{-6} mol l^{-1}; *N*=6), followed by incubation with 10^{-5} mol l^{-1} SNAP for 1 h before incubation with 10^{-4} mol l^{-1} SNAP for 30 min. Other details are as in Fig. 1.

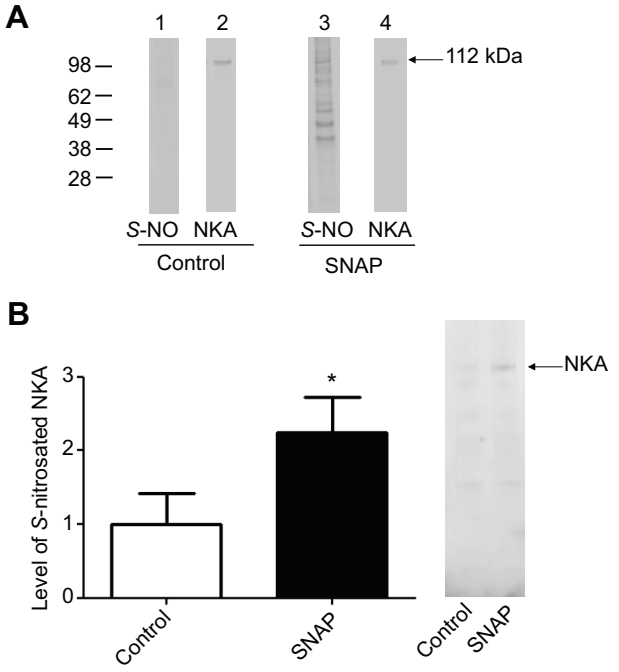

Fig. 7. Western blot analysis of *S*-nitrosated proteins and Na^+/K^+-ATPase (NKA) enzyme in the opercular epithelium of killifish (*N*=5). (A) Western blot analysis of a sample after the *S*-nitrosated protein (*S*-NO) assay: lane 1, control fish; lane 3, paired tissues incubated for 1 h with 10^{-4} mol l^{-1} SNAP where numerous *S*-nitrosated bands were detected; lanes 2 and 4 are identical to lane 1 and lane 3, respectively, but were reprobed with an anti-NKA antibody that recognized a protein at 112.2±1.1 kDa with a similar localization to that of an *S*-nitrosated band from lane 3. (B) Samples were subsequently purified by streptavidin pull-down to isolate *S*-nitrosated proteins. The presence of *S*-nitrosated NKA proteins and their increase following incubation with SNAP (*P<0.05) were confirmed by probing the membrane with an anti-NKA α-subunit antibody (α5).

S-nitrosation of NKA as an additional mechanism for NO action. The anti-NKA antibody recognized a major protein band at 112.2± 1.1 kDa and 111.4±1.1 kDa in opercular and branchial epithelium, respectively, although a lower molecular mass band at 82.7± 0.8 kDa was also recognized by the antibody in the gill. Isolation of the *S*-nitrosated proteins by streptavidin pull-down combined with western blot further confirmed the presence of *S*-nitrosated NKA among the *S*-nitrosated proteins in both opercular (Fig. 7B) and branchial (Fig. 8B) epithelium. Furthermore, the signal for *S*-nitrosated NKA proteins was significantly increased by incubation with the NO donor SNAP (Figs 7B and 8B). In some fish, endogenously *S*-nitrosated NKA proteins were detectable in the control tissue. Because of individual variability in the amount of NKA proteins, each individual tissue was solely compared with its paired control.

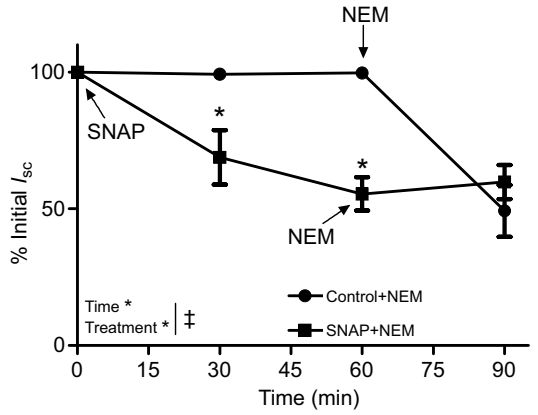

Fig. 6. Effect of NEM on I_{sc} with or without pre-incubation with SNAP. Preincubations were carried out with 10^{-4} mol l^{-1} SNAP, followed by incubation with 10^{-4} mol l^{-1} NEM (*N*=5). Other details are as in Fig. 1.

Independence from the adrenergic pathway

The α_2-adrenoceptor agonist clonidine induced an overall 56.8± 8.7% (*N*=5) decrease in I_{sc} that was dose dependent. Pre-incubation with yohimbine (10^{-4} mol l^{-1}) neutralized 85.6±2.0% (*N*=5) of the clonidine-induced I_{sc} decrease (10^{-4} mol l^{-1}) (Fig. 9A), confirming yohimbine as an α_2-adrenoceptor blocker in fish. Pre-treatment of tissues with yohimbine before addition of SNAP (10^{-6} mol l^{-1}) did not block the NO effect (initial I_{sc} decreased by 47.3±10.0%; *N*=3) (Fig. 9B).

In separate experiments, isoproterenol (10^{-6} mol l^{-1}), a β-receptor agonist, was added 60 min after SNAP (10^{-4} mol l^{-1}) (i.e. after the NO-induced decrease had reached its steady state) to confirm that epithelial preparations were still responsive to other stimuli and therefore that the NO-induced decrease of the I_{sc} could be neutralized. Isoproterenol alone induced a rapid 67.5±7.7% (*N*=5) increase in initial I_{sc} (Fig. 9C). Incubation with SNAP (10^{-4} mol l^{-1}) decreased the initial I_{sc} by 51.1±6.3% (*N*=5). Addition of isoproterenol (10^{-4} mol l^{-1}) after SNAP (10^{-4} mol l^{-1})

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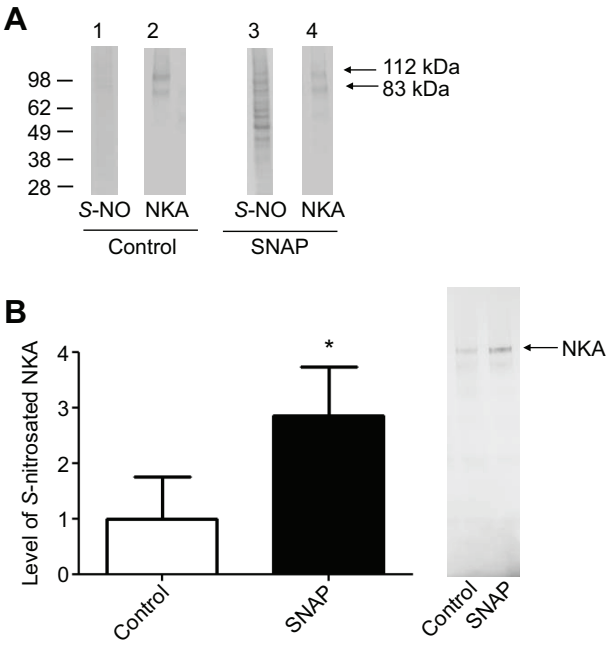

Fig. 8. Western blot analysis of *S*-nitrosated proteins and NKA enzyme in gill tissue of killifish (*N*=5). (A) Western blot analysis of samples after the *S*-nitrosated protein (*S*-NO) assay: lane 1, control fish; lane 3, paired tissues incubated for 1 h with 10^{-4} mol l^{-1} SNAP where numerous *S*-nitrosated bands were detected; lanes 2 and 4 are identical to lane 1 and lane 3, respectively, but were reprobed with an anti-NKA α-subunit antibody (α5) that recognized a major band at 111.4±1.1 kDa and a lower molecular mass band at 82.7± 0.8 kDa with a similar localization to that of an *S*-nitrosated band from lane 3. (B) Samples were subsequently purified by streptavidin pull-down to isolate *S*-nitrosated proteins. The presence of *S*-nitrosated NKA proteins and their increase following incubation with SNAP (*P<0.05) were confirmed by probing the membrane with an anti NKA α-subunit antibody (α5).

induced a rapid and full recovery of I_{sc}, with a 51.7±5.7% (N=5) increase of NO-induced I_{sc} (Fig. 9C).

DISCUSSION

Ion transport in fish gills has been the topic of numerous papers, and we have a good understanding of the essential elements involved at both cellular and molecular levels. For a few model species, there is also a good consensus about the endocrine factors responsible for major long-term adjustments, in particular in euryhaline species acclimating between fluctuating salinities. Fine tuning of ion transport, however, is an issue that has not received so much attention. One of the interesting candidates that may be involved in this is NO, which was investigated in the present study, using killifish opercular epithelium as a two-dimensional surrogate model of the teleost gill. We show for the first time that stimulation of endogenous NO production slowly but significantly inhibits Cl^- secretion in the opercular epithelium of seawater killifish. The effect was efficiently mimicked by SNAP, an appropriate NO donor when studying the *in vitro* effect of NO in fish. The study also brings new insights into the mechanisms of NO control of NaCl secretion in fish.

Endogenous and exogenous NO are involved in controlling NaCl secretion

In an initial paper, Tipsmark and Madsen (2003) found an inhibitory effect of exogenous NO on the *in vitro* enzymatic activity of NKA in the gill and kidney of brown trout and

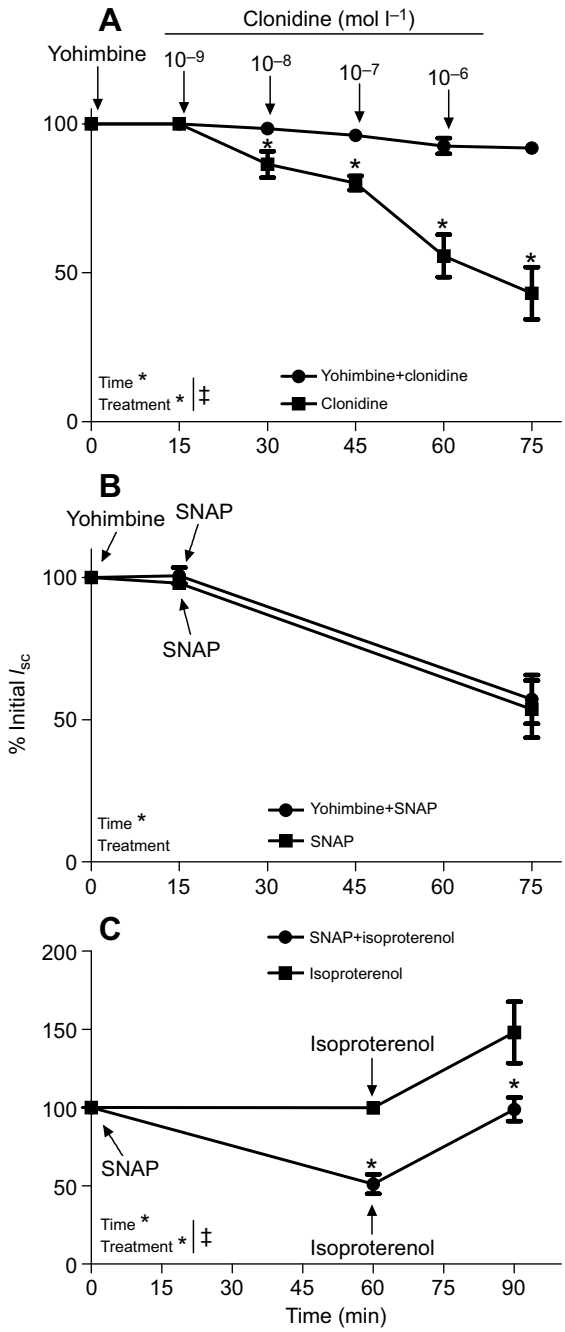

Fig. 9. Effect of adrenergic receptor agonists and blocker on I_{sc}. (A) Dose–response curve of the α_2-adrenoceptor agonist clonidine (10^{-9} to 10^{-6} mol l^{-1}) after pre-incubation with yohimbine (10^{-4} mol l^{-1}; N=5). (B) Effect of SNAP (10^{-4} mol l^{-1}) after pre-incubation with the α_2-adrenoceptor blocker yohimbine (10^{-4} mol l^{-1}; N=3). (C) Effect of the β-adrenoceptor agonist isoproterenol (10^{-4} mol l^{-1}) after incubation with SNAP (10^{-4} mol l^{-1}; N=5). Other details are as in Fig. 1.

suggested that NO might be involved in fine-tuning of ion transport in relation to seawater acclimation. Our study reveals that NO may optimize the regulation of ion transport by bridging rapid adrenergic and slow hormonal responses. Adrenergic responses are known to be rapid but not long-lasting (Marshall et al., 1998), whereas hormonal responses by cortisol, growth hormone and prolactin act slowly over days (Bern and Madsen, 1992; McCormick, 2001). NO effects appeared to be intermediate

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in onset and yet sustained over time, offering a potentially important intermediate mode of regulation for estuarine fish that frequently encounter salinity changes (Marshall, 2003).

Both NOS1 and NOS2 are expressed in fish gills (Mauceri et al., 1999; Barroso et al., 2000; Ebbesson et al., 2005; McNeill and Perry, 2006; Pederzoli et al., 2007; Porteus et al., 2015) making NO effects on ion transport possible. In the seawater-acclimated killifish, NOS1 was localized in both opercular (Evans, 2002) and branchial epithelium (Hyndman et al., 2006), while NOS2 localization has not been attempted yet. In this study, we observed that stimulation of endogenous NO production by L-arginine inhibited ∼48% of the initial I_{sc}, equivalent of Cl^- secretion, within 60 min (Fig. 1). To our knowledge, this is the first direct evidence that stimulation of endogenous NO production inhibits net Cl^- secretion in the opercular epithelium of seawater-acclimated killifish. Conversely, inhibition of NOS and thereby endogenous NO production by 10^{-4} mol l^{-1} L-NAME induced a ∼14% increase in I_{sc} (Fig. 2B), suggesting that Cl^- secretion is under tonic control by NO and that its regulation is not merely on–off but can be fine-tuned. Furthermore, the inhibitory effect of NO was reproduced by addition of an exogenous NO donor, SNAP. The fact that release of NO by SNAP reproduces the slow but strong and significant inhibition observed with L-arginine (Fig. 3) is a major finding. The effect of SNAP was elicited by NO, and not the donor molecule per se, as confirmed by pre-treatment of tissues with the NO scavenger PTIO, which neutralized the SNAP effect (Fig. 4). Even though the three concentrations of SNAP tested (10^{-6}, 10^{-5} and 10^{-4} mol l^{-1}) had a similar overall inhibitory effect (inhibition of ∼43% of the initial I_{sc}), they reached their maximal inhibition at different rates (Fig. 3), which may be the result of different accumulation rates into the tissue.

The ability of the fish opercular epithelium to respond to exogenous stimulation by NO and the tonic release of endogenous NO observed in this study is in agreement with a previous study by Evans et al. (2004). They used two other NO donors, sodium nitroprusside (SNP) and spermine NONOate (SPNO), at concentrations ranging from 10^{-10} to 10^{-5} mol l^{-1} and reported a ∼20% inhibition of I_{sc} at a critical threshold around 10^{-7} to 10^{-6} mol l^{-1}, SNP being the more potent in their experiments. They reported a concentration-dependent effect of NO donors, although – when reaching significance – all concentrations showed similar efficacy, as observed in our study. They did not report the time required to reach the maximal inhibition of I_{sc} with SNP and SPNO. Thus, as emphasized by Feelisch (1998), the choice of NO donor could be critical when evaluating and comparing dose–response relationships due to the various pathway(s) leading to formation of NO by the different compound classes. A dose–response experiment with regard to NO levels is hard to conduct and interpret because of diffusional issues, the slow response rate of the tissues to NO (SNAP; e.g. 60–90 min), the short life-time of NO molecules, and thus the uncertainty with regard to actual tissue NO levels. Our experiments were generally kept as short as possible to avoid degradation of the tissues and misinterpretation of the results. In another study, using intestinal tissue from the eel, Trischitta et al. (2007) reported a ∼30% reduction of initial I_{sc} within 60 min by addition of 10^{-4} mol l^{-1} L-arginine. They observed a maximal effect 1 h after addition of the NO donors SNP and 3-morpholinosydnonimine (SIN-1) at 10^{-3} and 10^{-4} mol l^{-1}, with approximately 40% and 30% reduction of initial I_{sc}, respectively. Thus, it can be concluded that the effects of NO-induced responses on ionic transport occur gradually and reach their maximum after approximately 1 h.

The NO effect is dependent on cGMP signalling pathways

The interaction of NO with sGC leads to one of the best established pathways for the biological effects of NO (Hill et al., 2010). Briefly, the specific interaction of NO with the sGC haem activates sGC, which generates cGMP that in turn mediates the biological action of NO (Denninger and Marletta, 1999). The two inhibitors of sGC used in this study, MB and ODQ, neutralized >80% of the SNAP-mediated decrease in I_{sc} (Fig. 5), suggesting that NO is predominantly acting through the cGMP pathway, though other pathways to a lower extent could add to the NO signalling mechanism. Because of the potential direct inhibition of NOS by MB and its less-specific capacity to inhibit sGC (Mayer et al., 1993), we also used ODQ, a more specific inhibitor of sGC, and confirmed the observation made with MB. To our knowledge, only one previous study used ODQ as a sGC inhibitor in fish: Trischitta et al. (2007) found that the effect of 10^{-3} mol l^{-1} SNP was 50% reduced by pre-treatment with ODQ. Quantitative interpretation of the observed ODQ effect, however, has to take into account that ODQ and NO are competitors regarding the action on sGC, and Lies et al. (2013) showed that NO at high concentrations can overcome the inhibitory effect of ODQ on sGC, meaning that the lack of effect of ODQ does not exclude cGMP signalling or prove cGMP independence. Hence, to avoid misinterpretation, SNAP was first injected at an intermediate concentration (10^{-5} mol l^{-1}) and subsequently increased to reach the maximal concentration used in the study (10^{-4} mol l^{-1}). Addition of SNAP 10^{-4} mol l^{-1} slightly but significantly decreased the I_{sc} in the control tissues pre-incubated with ODQ (Fig. 5B) probably showing the threshold concentration where the inhibitory effect of ODQ on sGC is overcome by NO. However, taken together, the results from the ODQ and MB experiments allowed us to conclude that the main pathway of NO action is via cGMP. This is in agreement with Tipsmark and Madsen (2003), where the whole-tissue cGMP concentration increased in response to NO donors and where the analogue db-cGMP inhibited NKA activity in a similar manner to NO. This is also consistent with the general conception that sGC is among the most sensitive targets of NO and that NO is a physiological regulator of cGMP production (McDonald and Murad, 1996).

S-Nitrosation of transport proteins as a cellular target for NO action

Several different classes of membrane proteins have been identified as potential targets for *S*-nitrosation (Stamler et al., 2001; Hess et al., 2005), including three important ion transporters present in branchial and opercular epithelia: NKA (Sato et al., 1995; Jaffrey et al., 2001; Althaus et al., 2011), CFTR (Gaston et al., 2003; Matalon et al., 2003; Guo et al., 2008) and NKCC (Herrera et al., 2006). It is well known that *S*-nitrosation may induce modification of the activity and function of the targeted proteins. Sato et al. (1995) observed inhibition of NKA activity by several NO donors in porcine cerebral cortex and suggested *S*-nitrosation of the -SH group at the active site of the enzyme as a potential mechanism. Additionally, Jaffrey et al. (2001) and Althaus et al. (2011) observed *S*-nitrosation of NKA proteins by NO donors in the H441 human bronchiolar epithelial cell line and in rat brain, respectively. In fish, it is known that conditions eliciting NO formation also increase levels of *S*-nitrosated compounds in various tissues (Sandvik et al., 2012), but only a few specific proteins have so far been identified as *S*-nitrosated, including phospholamban (Garofalo et al., 2009). We suspected *S*-nitrosation of the -SH group of the NKA enzyme as a secondary NO-induced mechanism to reduce NKA activity, which could contribute to the decrease of ionic

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transport. Indeed, we provide the first evidence of *S*-nitrosation of proteins by a NO donor in opercular and branchial epithelium tissues of fish. After 1 h incubation of branchial and opercular epithelia *in vitro* with SNAP (10^{-4} mol l^{-1}), a number of *S*-nitrosated proteins were detected (Figs 7B and 8B). Interestingly, NO release by SNAP increased *S*-nitrosation of a protein(s) around 112 kDa, where the transport protein NKA was also detected (Figs 7A and 8A). We confirmed the presence and NO-induced increase of *S*-nitrosated NKA using streptavidin pull-down combined with western blot (Figs 7B and 8B). This first demonstration of *S*-nitrosated NKA in fish supports that *S*-nitrosation by NO may be an additional way of regulating the protein, but the detailed implications of this modification for NKA activity and epithelial ion transport must await future investigations. The degree of *S*-nitrosation of transport proteins is probably in a dynamic equilibrium and could contribute to the rapid regulation of ion transport. A rapid increase in gill NKA activity, independent of translational and transcriptional processes, occurs during the first hours of SW transfer in *F. heteroclitus* (Towle et al., 1977; Mancera and McCormick, 2000), *Mugil cephalus* (Hossler, 1980) and *Oreochromis mossambicus* (Hwang et al., 1989). The mechanism (s) for this rapid increase of NKA activity remains largely unexplained and may involve the reversal of NO-induced nitrosation. We showed that alteration of -SH groups of proteins may decrease the short-circuit current (Fig. 6). Yet, as alkylation by NEM is chemically different from *S*-nitrosation by NO donors, we cannot certify that *S*-nitrosation of NKA proteins would have participated in the decrease of the I_{sc} and, if so, to what degree. Similarly, the capacity of endogenous NO to *S*-nitrosate transport proteins needs to be assessed. However, the dual mechanisms by which NO can affect ion transport make it an important 'bridge' type of regulator.

Interference of NO with hypotonic and adrenergic pathways?

Mediation of hypotonic-induced decreases in Cl^- secretion is not fully understood, and a potential role of NO has been suggested by Marshall et al. (2005). Our study suggests a secondary role of NO in the 'shut down' of Cl^- secretion and in contributing to the inhibition when fish encounter a marked drop in salinity. A 61 mOsm kg^{-1} decrease in osmolality from the normal 305 mOsm kg^{-1} induced a ∼75% decrease in I_{sc} within 20 min (Fig. 2). These results are consistent with Marshall et al. (2000), where a reduction of 40 mOsm kg^{-1} decreased I_{sc} by ∼60% in 40 min. Tissues pre-treated with PTIO (Fig. 2C) present smaller reductions in I_{sc} than control tissues, suggesting a small but significant involvement of NO in the shut-down of Cl^- secretion during hypotonic exposure. Supplementary observations were made when L-NAME was added after the hypotonic shock, with a ∼25% significant increase of hypotonic-induced I_{sc} from 30 to 90 min (Fig. 2A), suggesting that NO production during the hypotonic shock contributes to the inhibition, yet NO effects were delayed relative to the rapid initial neuronal response (Marshall et al., 1998). This is confirmed by Fig. 2B, as blocking of NO production before hypotonic shock eliminated the increase observed in Fig. 2A. This may reflect an involvement of NO in cell volume regulation with stimulation of NOS by hypotonic swelling. In humans, it has been shown that hypotonic swelling promotes NO release in cardiac ventricular myocytes (Gonano et al., 2014). Marshall et al. (2005) connected cell swelling and inhibition of Cl^- secretion in opercular epithelium of seawater-acclimated killifish, but the mechanism of the volume regulatory response is not yet clear and will be an interesting area for future investigation.

The neurally mediated decrease of Cl^- secretion was first observed by Marshall et al. (1998). They showed that seawater-acclimated killifish decrease the Cl^- secretion rate through the action of the sympathetic nervous system mediated by α_2-adrenoceptors via inositol 1,4,5,-trisphosphate (IP3) and intracellular [Ca^{2+}], and that the response can be mimicked by clonidine and blocked by yohimbine. This was confirmed in our study, where clonidine (10^{-9} to 10^{-6} mol l^{-1}) induced a ∼13–57% decrease of initial I_{sc} and where pre-incubation with yohimbine prevented ∼85% of the induced decrease (Fig. 9A). NOS1, the neuronal isoform of NOS, is a good candidate for the Ca^{2+}-dependent adrenergic pathway, as its presence has been shown both by its immunoreactivity in the killifish gill nerve fibres (Hyndman et al., 2006) and the opercular epithelium adjacent to chloride cells (Evans, 2002) and by its Ca^{2+}-dependent activity (Øyan et al., 2000; Andreakis et al., 2011). Yohimbine was used to block the adrenergic receptors mediating the neural control of NaCl secretion, but it did not prevent the inhibition of Cl^- secretion induced by NO (Fig. 9B). Hence, the pathway for NO action seems to be independent of the adrenergic pathway even though they are likely to converge at the Ca^{2+} mobilization level. Furthermore, the stimulation of Cl^- secretion by isoproterenol, a β-adrenoceptor agonist, after SNAP (Fig. 9C) shows that the effects were additive and that NO does not act through or affect β-adrenoceptors. The capacity to overcome NO inhibition by adrenergic stimuli is an interesting finding and could be of use in pharmacological studies.

Perspectives and significance

This study documents an intermediate modulatory role of NO in controlling NaCl secretion in the seawater-acclimated killifish that is predominantly mediated by the cGMP pathway, but some involvement of *S*-nitrosation of ion transport proteins is also suggested. The study documents *S*-nitrosation of proteins after incubation with the NO donor SNAP and highlights NKA as a target. It is also of interest to investigate other targets of *S*-nitrosation involved in ion transport. In the estuarine killifish, which daily encounters salinity fluctuations, modulation of ion secretion seems most likely to be a result of combined actions of several different signalling molecules and mechanisms with different time courses and longevities.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.B.J. and S.S.M. conceived the study; L.G., F.B.J., S.S.M. and W.S.M. designed the experiments; L.G. and W.S.M. performed the experiments; L.G. analysed the data and drafted the manuscript; F.B.J., S.S.M. and W.S.M. revised the manuscript.

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