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A model system using confocal fluorescence microscopy for examining real-time intracellular sodium ion regulation

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ABSTRACT

The gills of euryhaline fish are the ultimate ionoregulatory tissue, achieving ion homeostasis despite rapid and significant changes in external salinity. Cellular handling of sodium is not only critical for salt and water balance but is also directly linked to other essential functions such as acid–base homeostasis and nitrogen excretion. However, although measurement of intracellular sodium ($[Na^+]_i$) is important for an understanding of gill transport function, it is challenging and subject to methodological artifacts. Using gill filaments from a model euryhaline fish, inanga (*Galaxias maculatus*), the suitability of the fluorescent dye CoroNa Green as a probe for measuring $[Na^+]_i$ in intact ionocytes was confirmed via confocal microscopy. Cell viability was verified, optimal dye loading parameters were determined, and the dye–ion dissociation constant was measured. Application of the technique to freshwater- and 100% seawater-acclimated inanga showed salinity-dependent changes in branchial $[Na^+]_i$, whereas no significant differences in branchial $[Na^+]_i$ were determined in 50% seawater-acclimated fish. This technique facilitates the examination of real-time changes in gill $[Na^+]_i$ in response to environmental factors and may offer significant insight into key homeostatic functions associated with the fish gill and the principles of sodium ion transport in other tissues and organisms.

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Euryhaline fish, those capable of inhabiting a wide range of salinities, are archetypal ion regulators. Some fish are able to maintain salt and water balance even as external osmolalities range from near zero (dilute freshwater, FW) to hypersaline waters. To a large degree, this ability is conferred through the actions of the fish gill. The branchial epithelium of fish is characterized by its large surface area, high blood perfusion, and small diffusive distances, features critical for osmoregulation, acid–base homeostasis, and nitrogen excretion [1]. All of these processes are linked, directly or indirectly, to sodium transport. In seawater (SW) fish gills, for example, the basolateral sodium pump (Na^+,K^+–ATPase, NKA) translocates intracellular sodium to intercellular spaces and generates a transepithelial electrochemical gradient that drives sodium ion secretion through leaky paracellular junctions [2]. In FW, where sodium is actively absorbed from the surrounding water, sodium transport mechanisms are less clear. This is partly a consequence of variations in cellular and molecular composition between different species but is also due to uncertainty regarding the thermodynamics of sodium transport [3–5].

For many fish, the apical sodium hydrogen exchanger (NHE) is thought to be the key entity in achieving sodium uptake in FW. However, standard transport models dictate that an intracellular sodium concentration ($[Na^+]_i$) lower than that of the surrounding water is required to facilitate sodium influx [4]. Measurements indicate that the $[Na^+]_i$ of FW fish gill cells fails this criterion [6–9]. However, determination of $[Na^+]_i$ is potentially compromised by loss of polarity through cell isolation (see, e.g., Ref. [9]) or through other methodological artifacts [8]. Recently, the discovery of a functional metabolon consisting of NHE and Rh proteins (a group of epithelial ammonia transporters) has offered an alternative model that is thermodynamically more favorable [10]. There remains, however, the need for methods to measure real-time sodium concentrations inside gill cells. Such techniques would

Abbreviations used: FW, freshwater; SW, seawater; NKA, Na^+,K^+–ATPase; NHE, sodium hydrogen exchanger; $[Na^+]_i$, intracellular sodium concentration; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

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enable identification of low sodium intracellular microenvironments thought to be key in achieving apical uptake in FW fish [4,5].

CoroNa Green is a sodium-specific dye that is insensitive to changes in potassium concentration [11] and that has been successfully used for measurement of $[Na^+]_i$ in a variety of experimental systems (e.g., rat cardiomyocytes [12], plant root–tip cells [13]). The current study validated the utility of CoroNa Green for measuring $[Na^+]_i$ in the cells of gill filaments of the model teleost fish species inanga (*Galaxias maculatus* Jenyns, 1842). The gill filament technique involves the removal of a gill arch with filaments intact. This is advantageous because maintaining gill cells in their natural setting represents a more physiologically realistic approach to examining ion transport [14]. When applied to model species, this represents a powerful tool for investigating cellular ion transport. Inanga can be considered Krogh models for studying ion transport [15] in that they exhibit an extreme capacity to withstand salinity change [16–18] by virtue of their amphidromous lifestyle that allows them to move freely between waters of distinct salinities. Furthermore, the cellular machinery they use to achieve sodium ion homeostasis appears to be conserved relative to other fish species and, as such, closely mimics sodium transport mechanisms in other animal cells [17]. Consequently, an understanding of sodium ion metabolism in inanga may have applicability to the study of ion transport across phyla.

Materials and methods

Animals and salinity acclimation

Adult inanga (*G. maculatus*, ~0.5–2 g) were collected from coastal streams in the Canterbury region of New Zealand. Fish were held in flowing FW (315–330 μM Na^+, pH 6.8–7.2) with constant aeration at 15 °C on a 12:12 day/night cycle for at least 2 weeks prior to experimentation. Animals were fed ad libitum daily on commercial flake food throughout this period.

For salinity acclimation, inanga were gradually acclimated to 50% SW (17‰, added via freshly collected SW sourced from Lyttelton Harbour, Canterbury, New Zealand) over a 1-week period (increase in salinity of 7% per day) and were then held at this salinity for a week. A second group of inanga was taken through this exact protocol except that after a week the inanga were subjected to similar salinity changes (7% per day) up to 100% SW (33‰), where they were also held for a week prior to experimentation. All procedures were approved by the University of Canterbury animal ethics committee.

Tissue preparation

Following euthanasia (cephalic blow followed by severing of the spinal cord), gill arches were excised and placed directly into an aliquot of water to which the inanga had been acclimated (FW, 50% SW, or 100% SW). Gill mucus was left intact to help maintain the gill microenvironment and ensure prolonged survival of the filaments post-dissection. Immediately before use, each arch was carefully blotted with tissue paper to remove the protective mucus and facilitate dye loading.

Fluorescent dyes

CoroNa Green AM, MitoTracker Orange, and LysoTracker Red (Life Technologies, Carlsbad, CA, USA) were reconstituted to a concentration of 1 mM with anhydrous dimethyl sulfoxide (DMSO) and stored aliquoted at −20 °C. Optimal CoroNa Green loading conditions (10 μM in either FW, 50% SW, or 100% SW, 10 min) were determined by exposing gill tissue to various dye concentrations (0.5–20 μM) for 10 min and loading 10 μM dye for various incubation times (1–45 min). Mitochondria were labeled with MitoTracker Orange (0.5 μM, 10 min), whereas lysosomes were labeled with LysoTracker Red (0.5 μM, 10 min).

Imaging

Confocal imaging of CoroNa Green and MitoTracker Orange was performed with a DMIR inverted microscope (Leica model SP5, Wetzlar, Germany) using 20× NA 0.7 and 63× NA 1.3 glycerol immersion lenses with 488- and 561-nm excitation and 500- to 580-nm and 571- to 671-nm emission windows for CoroNa Green and MitoTracker Orange, respectively. LysoTracker Red imaging was similar to that described for MitoTracker Orange except with an emission window of 580–620 nm. CoroNa Green images were quantified with Leica Microsystems LAS AF Lite software and corrected for autofluorescence. Images were collected in a 512 × 512-pixel format with 8-bit resolution and averaged twice via the software-selected repeated line scan mode to improve the signal-to-noise ratio. All validation and measurements of $[Na^+]_i$ were made in ionocytes lining the lamella identified by MitoTracker Orange labeling. There was no attempt to differentiate between different types of ionocytes should these exist in inanga gills. For optimal imaging, a single gill arch was removed from the gill basket, carefully blotted with tissue paper to remove the surrounding mucus, and placed in CoroNa Green dye (10 μM, 10 min). The gill arch was then rinsed in a fresh change of acclimation water, placed into a 50-mm FluoroDish (World Precision Instruments) cell culture dish with a small volume of the acclimation water, and covered with a 32-mm diameter round coverslip to help immobilize the tissue and prevent desiccation. However, because the cells were not adhered to the FluoroDish, movement sometimes occurred.

Viability of tissue

Because CoroNa Green AM's ability to bind sodium relies on esterase cleavage of the AM moiety, a standard test for cell viability, the dye can also be used to assess cell viability post-dissection. Gills were excised with the time of death being noted and were loaded and visualized at various intervals to determine viability.

Determination of dissociation constant

To determine the dissociation constant (K_d), an in situ response calibration was performed by measuring fluorescence corresponding to a series of precisely manipulated external sodium concentrations. Fifteen calibration solutions, based on a Hepes–Cortland saline buffer [19] and ranging in sodium concentration from 0 to 300 mM (sodium concentrations confirmed via flame photometry), were made with choline chloride and choline bicarbonate used as sodium salt substitutes to ensure that the concentrations of other ions and osmolality (~300 mmol/kg) of all solutions were similar [20]. All solutions were adjusted to pH 7.7 using HCl. Sodium transport modifiers (3 μM gramicidin, 10 μM monensin, and 100 μM ouabain; Sigma) were added to the solutions to equilibrate $[Na^+]_i$ with the external sodium concentration.

Fluorescence intensities for selected regions of interest in a minimum of 6 cells from three separate replicate runs were measured with the Leica software. For each calibration solution, these intensities were averaged and then normalized, with this protocol repeated for each acclimation. To determine the K_d, the log of the actual sodium concentration of the calibration solution (log $[Na^+]$) was plotted against the log of the normalized fluorescence intensity of each calibration sample [21,22]. Linear regression was

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