Sodium entry pathways in isolated goldfish hair cells

by

Diane Elizabeth Ronan

B.S., Electrical Engineering
Massachusetts Institute of Technology, 1992

Submitted to the Harvard-M.I.T. Division of Health Sciences and Technology in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Abstract

Hair cells are the exquisitely-sensitive mechanosensory transducers of the inner ear. While the electrophysiology of hair cells has been extensively studied, little is known about the vital background processes that maintain the steady-state intracellular ionic environment. This study explored Na⁺-coupled ion transport processes in isolated goldfish hair cells both in the steady state and in response to perturbations. Intracellular Na⁺ concentration ([Na⁺]i) and pH (pHᵢ) were measured using the fluorescent probes SBFI and BCECF, respectively. The total steady-state Na⁺ entry, determined by measuring the initial rate of increase in [Na⁺]i during inhibition of Na⁺,K⁺-ATPase, was 9 mM/min, a rate higher than in many other epithelial cells involved in rapid ion transport. To uncover the entry pathways for such a high Na⁺ influx, pharmacological agents and manipulations of extracellular composition were used to dissect apart the contributions of various transporters or channels. A combination of Na⁺ entry via transduction channels, the Na⁺/H⁺ exchanger, and the Na⁺/Ca²⁺ exchanger accounted for 3/4 of the total steady-state Na⁺ entry. Consistent with a significant component via the Na⁺/Ca²⁺ exchanger, nifedipine, a blocker of L-type calcium channels, also reduced the Na⁺ entry rate. Since the Na⁺/H⁺ exchanger in goldfish hair cells, in contrast to many other cell types, displayed significant activity in the steady state, interactions between regulation of [Na⁺]i and pHᵢ were examined during recovery from intracellular acid loads. The rate of Na⁺ entry or acid extrusion via the Na⁺/H⁺ exchanger increased by a factor of 6 during recovery from an acid load. In most cells, [Na⁺]i doubled after the acid load and subsequently recovered, even though the Na⁺,K⁺-ATPase was not exogenously inhibited. These results indicate that intracellular acidification inhibits the Na⁺,K⁺-ATPase, and therefore poses a potential conflict for maintenance of intracellular Na⁺ homeostasis. Although hair cells were traditionally described as passive resistors, this thesis demonstrates that goldfish hair cells have a significant metabolic load, even in the steady state, arising from the activities of specific Na⁺-coupled transporters and channels. Under certain pathophysiological circumstances, such as ischemia or acoustic trauma, increased Na⁺ influx via these pathways may overwhelm the ion-homeostatic capabilities of hair cells.

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How can I ever thank the two people who have given me more love than I ever thought possible? Mom and Dad, you always believed in me and showed me how proud you were of me. In the hard times, when I wasn’t sure I had what it took to make it through, I drew strength from your love. You both will always be the beacons in life. Mom, you say that I got my brains from Dad, but you are the wisest woman I know. You are an example of how we all should live our lives – with kindness and purity of heart. Dad, it has been 10 years since you passed away and I still feel the empty space in my heart. You are the reason I wanted to go to MIT. You encouraged me to become an engineer and you led by your unwavering example of a hard-working family man.

Ian, my fiancée, you have brought much joy, laughter, and peace into my life. You have been incredibly patient and giving. I am so excited about the new life we are about to embark on together.
In loving memory of my hero, my father
Kevin Thomas Ronan
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The steady-state Na\(^+\) entry rate measured in goldfish hair cells is consistent with “leak” currents in whole-cell patch clamp studies. Transduction channels represent a significant component of steady-state Na\(^+\) entry in vitro. The measured Na\(^+\) entry via transduction channels is consistent with estimates of steady-state conductance and may be similar to the in vivo rate.

Goldfish hair cells, unlike guinea-pig outer hair cells, have steady-state activity of the Na\(^+\)/H\(^+\) exchanger. Steady-state Na\(^+\)/H\(^+\) activity may be a hallmark of highly active cells. The Ni\(^{2+}\)-sensitive component is most likely the Na\(^+\)/Ca\(^{2+}\) Exchanger.

As opposed to cardiac myocytes, goldfish hair cells do not have a significant component of steady-state Na\(^+\) entry via TTX-sensitive Na\(^+\) channels. K\(^+\)-depolarization rapidly decreased intracellular [Na\(^+\)]; removing divalent cations caused very large increases in [Na\(^+\)]. Carbenoxolone had unexpected effects on steady-state [Na\(^+\)]; and Na\(^+\) entry.

We have accounted for ¼ of total steady-state Na\(^+\) entry.

What pathways might account for the remaining ¼ of steady-state Na\(^+\) entry?

Fish hair cells may be better able to deal with Na\(^+\) loads than are mammalian hair cells.

Captions, Tables, and Figures

Chapter 4: Interactions between [Na\(^+\)]\(_i\) and pH during an imposed intracellular acid load in isolated goldfish hair cells

Abstract

Introduction

Methods and Materials

Solutions and chemicals

Drugs

Hair cell dissociation

Loading of fluorescent ratio ion indicators

Measurements of intracellular Na\(^+\) or pH

Calibration

Rate of proton extrusion via the Na/H exchanger

Steady-state Na\(^+\) entry rate

Statistics

Results

pH\(_i\) response to acid loading

[Na\(^+\)]\(_i\), response to acid loading: No exogenous pump inhibition

Na\(^+\) response to simultaneous pump inhibition and acid load

Discussion

Comparison of steady-state and acid-loaded rates of the Na\(^+\)/H\(^+\) exchanger among different cell types

Other cell types also exhibit increases in [Na\(^+\)]\(_i\) during recovery from an acid load
CHAPTER 1: Introduction

This thesis describes the vital background processes of Na\(^+\) ion homeostasis in hair cells. Animal cells typically maintain a low intracellular Na\(^+\) concentration. The resulting electro-chemical gradient for Na\(^+\) ions across the plasma membrane provides the energy necessary for Na\(^+\)-coupled uphill transport of metabolites and ions. A third or more of a cell’s resting ATP turnover is consumed by the Na\(^+\),K\(^+\)-ATPase, or Na\(^+\)-pump, to extrude Na\(^+\) ions (Clausen et al., 1991). Cells with similar intracellular Na\(^+\) ion concentrations may have vastly different steady-state rates of passive Na\(^+\) influx balanced by active Na\(^+\) extrusion via the Na\(^+\),K\(^+\)-ATPase. Generally cells with higher metabolic demand have higher steady-state Na\(^+\) entry rates (Lechene, 1988).

Hair cells, the primary sensory cells of auditory and vestibular organs, were classically described as passive variable resistors with little metabolic demand (Davis, 1968). The discovery of extremely rapid sodium turnover in sensory hair cells of the goldfish (Mroz et al., 1993a) thus poses a challenge to our understanding of these cells’ metabolic demands. Hair cells from goldfish were found to have Na\(^+\) turnover rates as fast as those of mammalian epithelial cells engaged in rapid ion transport, and orders of magnitude faster than those of other cell types in several species.

This thesis explores both the sources and the significance of the rapid sodium turnover in this primary sensory cell. Methods for optically measuring intracellular pH and Na\(^+\) concentrations with fluorescent dyes were optimized for these hair cells, then used in combination with pharmacological treatments that block or activate particular
transport processes to determine the dynamics and relative contributions of sodium-coupled transport processes. Knowledge of the major routes of resting Na\(^+\) entry can help define the physiological role of Na\(^+\) homeostasis for hair cell function in vivo. Perturbations of some of these resting pathways were used to define boundaries for the short-term ion-homeostatic capabilities in hair cells and to predict which physiological situations in vivo may pose a problem for intracellular Na\(^+\) regulation.

**Organization of Thesis**

Chapter 2 describes the background and significance of Na\(^+\) ion homeostasis for hair cells. Chapters 3 and 4 are formatted as individual journal articles, each with its own introduction, methods, results, and discussion, followed by tables and figures. Chapter 3 is titled “Sodium entry pathways in isolated goldfish hair cells”. This chapter describes Na\(^+\) entry pathways and their relative quantitative contributions to total steady-state Na\(^+\) entry. Chapter 4 is titled “Interactions between [Na\(^+\)]\(_i\) and pH during an imposed intracellular acid load in isolated goldfish hair cells”. This chapter describes the ability of the cell’s Na\(^+\) and pH regulatory processes to deal with a large perturbation, an intracellular acid load. Chapter 5 summarizes the main conclusions and the implications of the work carried out in this thesis.
CHAPTER 2: Background

Overview of hair cell anatomy and physiology

Hair cells detect motions of their apically-located hair bundles.

Hair cells are the exquisitely sensitive sensory transducers of the inner ear and lateral-line organ. They are the first stage in transmission of information to the brain about acoustic vibrations due to sound or mechanical signals relating to movements of the body in space.

In all sensory hair-cell organs, a chain of processes in the hair cell transduces acoustic or mechanical stimuli into nerve action potential signals to the brain. First, mechanical stimulation results in tilting of the apical hair bundles relative to the cells’ apical surfaces (Figure 1). This tilting changes the probability that mechanically-sensitive transduction channels in the individual stereocilia of the hair bundles are open. An unusual fluid bathes the apical membrane, including the hair bundle. This fluid, called endolymph, has a high K⁺ concentration, unlike most extracellular fluids, and a positive electrical potential (10 mV in the fish saccule, 80 mV in the mammalian cochlea) with respect to body ground, whereas the cell interior has a negative potential. Therefore a large driving force exists for flow of K⁺ current into the cell via transduction channels. The magnitude of the transduction current due to this driving force depends on the conductance, which is proportional to the number of open channels. Absent mechanical stimulation, about 10% of these channels are open. Thus a standing or “silent” current (Zidanic and Brownell, 1990), composed mostly of K⁺ ions, flows into the hair cell via these transduction channels. Mechanical stimuli modulate the open probability around
this baseline value (Corey and Hudspeth, 1979). The stimulus-driven transduction currents modulate the voltage difference across the basolateral surface of the hair cell. These voltage changes then modulate basolateral hair-cell calcium currents which cause calcium-dependent neurotransmitter release. This neurotransmitter activates action potentials in the primary afferent neurons, which transmit information to the brain.
Figure 1: Basic transduction process in a hair cell. At the top of the cell protrudes a hair bundle composed of individual actin-filled stereocilia. Fine filaments, called tip links, connect near the tips of individual stereocilia. Motion of the hair bundle in the direction towards the tallest stereocilium stretches the tip links, which are thought to directly open mechanically-gated ion channels (Corey and Hudspeth, 1983), called transduction channels, located in the stereocilia. A tight junction barrier between hair cells and supporting cells (not shown here) separates the fluid that bathes the top of the cell from the basolateral membrane. The top of the hair cell faces a fluid with an unusual ion composition with high $K^+$ and low $Na^+$ concentrations. When transduction channels are opened, a cation current, composed mainly of the abundant ion $K^+$, flows into the hair cell and depolarizes the membrane potential (i.e. makes it more positive relative to the rest potential of $-70$ mV). This depolarization increases $Ca^{2+}$ influx via basally-located voltage-gated calcium channels ($gCa^{2+}$). Local increases in $[Ca^{2+}]_i$ activate a cascade of processes leading to neurotransmitter release. In addition, basolaterally located $K^+$ channels ($gK^+$) are activated, allowing $K^+$ ions to flow out of the cells down their electro-chemical gradient and repolarizing the membrane potential back towards the resting level. The $K^+$ and $Ca^{2+}$ channels are very close to each other in reality (Roberts et al., 1990), but for clarity they are separated in this diagram.
Hair cells were traditionally described as passive resistors with little metabolic demand.

Since the transduction channels are cation-selective but otherwise nonspecific among small cations (Corey and Hudspeth, 1979), the predominance of K⁺ both inside and outside the apical hair-cell membrane means that most of the cation current passing inward through transduction channels is carried by K⁺ ions.

The extracellular surfaces of the basolateral membranes of hair cells face a fluid, called perilymph, with the high Na⁺ and low K⁺ characteristic of most animal extracellular fluids. Across the basolateral membranes, the electrochemical potential difference for K⁺ favors exit of that ion. From the perspective of the hair cell, the flow of K⁺ ions into its apex and out through its basolateral surfaces is entirely passive and therefore poses little metabolic demand. Cell types other than the hair cells (Kikuchi et al., 2000) expend energy to recirculate K⁺ ions from perilymph back to endolymph in order to maintain the high K⁺ concentration of endolymph. Indeed, these cells, which are termed dark cells (in the vestibular system), ionocytes (in fish) and strial marginal cells (in the cochlea), stain very darkly for Na⁺,K⁺-ATPase (Spicer et al., 1990; ten Cate et al., 1994; Zuo et al., 1995). Thus hair cells have been described as essentially variable resistors with only minimal metabolic demands (Davis, 1965, 1968).

Goldfish hair cells have a remarkably high Na⁺ turnover rate.

Mroz et al (1993a) measured steady-state rates of Na⁺ entry in isolated goldfish hair cells. Surprisingly, these hair cells had a very rapid Na⁺ turnover time, which was as fast as in mammalian renal proximal tubule cells whose main function is rapid Na⁺ ion transport (Harris et al., 1986). Rapid Na⁺ turnover is an energy-consuming process which
requires pumping Na\(^+\) ions out of the cell via the Na\(^+\),K\(^+\)-ATPase, and thus this result conflicts with the idea of hair cells as passive resistors. Therefore we must consider the physiological roles of Na\(^+\) turnover in hair cells. The next two sections describe the significance of Na\(^+\) turnover for the function of any cell in general, and for hair cells in particular.

*Significance of Na\(^+\) turnover in a cell*

**Intracellular Na\(^+\) homeostasis is essential for the function of most animal cells.**

It has been long recognized that the composition of the extracellular fluids is precisely controlled (Bernard, 1878). Thus individual cells are not normally exposed to large fluctuations in their extracellular environment. Yet all cells have homeostatic mechanisms to control their intracellular milieu. Cells are surrounded by a plasma membrane made up of a phospholipid bilayer studded with many proteins and other macromolecules, which make the membrane permeable to specific solutes. Cellular ion homeostasis involves the combined operation of these transport proteins, which shuttle ions and metabolites back and forth between the cell interior and the external environment to regulate the steady-state ion contents, membrane potential, cell volume and pH (Shultz, 1987; Hoffmann and Simonsen, 1989). The specialized processes of different cell types, such as secretion, absorption, and digestion, have optimal rates that depend on a precisely-controlled intracellular ionic environment.
Almost all animal cells maintain a high intracellular potassium concentration ([K\(^+\)]_i > 120 mM) and a low intracellular sodium concentration ([Na\(^+\)]_i < 20 mM). Combined with a negative resting potential, there is a large electro-chemical driving force for Na\(^+\) entry and K\(^+\) exit across cell plasma membranes that face typically high-Na\(^+\), low-K\(^+\) extracellular fluids. The ubiquitous Na\(^+\),K\(^+\)-ATPase (Skou, 1957), or Na\(^+\)-pump, uses ATP to actively pump Na\(^+\) ions out of and K\(^+\) ions into the cell.

Both the high intracellular K\(^+\) and the low Na\(^+\) are important to animal cells. First, intracellular enzymes evolved with K\(^+\) as the major intracellular monovalent cation. The reasons for selection of K\(^+\) over Na\(^+\) may have to do with differences between the two ions in their effects on cellular water structure, protein stability, chromosome structure (Parkinson et al., 2002), and enzyme activity (Hochachka and Somero, 2002 pp 223-226). Second, influx of Na\(^+\) ions via channels, cotransporters and antiporters is at the core of many differentiated cell functions, such as nutrient absorption in the gut, action potentials in neurons, and gland secretion, as well as general cellular functions such as pH and volume regulation (Lechene, 1988). The large electrochemical gradient of Na\(^+\) across the plasma membrane of animal cells energizes Na\(^+\)-coupled uphill transport of metabolites and ions. For example, most cells have an intracellular pH which is more alkaline than would be expected based on a passive distribution across the plasma membrane (Roos and Boron, 1981). Either Na\(^+\)/H\(^+\) exchange or Na\(^+\),HCO\(_3\)\(^-\) cotransport are used by cells to extrude acid or import base equivalents against this gradient.

This use of the Na\(^+\) gradient as a secondary energy source for transport across plasma membranes in animal cells provides a cellular “Na\(^+\) economy”, in place of the
analogous proton economy of eubacteria and of mitochondria, which descended from bacterial cells (Margulis, 1976).

**Different cell types have different rates and pathways of steady-state Na\(^+\) entry.**

Despite having similar intracellular Na\(^+\) ion concentrations, cell types can differ by two orders of magnitude or more in their steady-state rates of Na\(^+\) influx and of Na\(^+\),K\(^-\)-ATPase, whose balance determine [Na\(^+\)]. Even within a cell type the rate of the Na\(^+\),K\(^-\)-ATPase can vary to 3-4 fold depending on intra- or extracellular signals (Lechene, 1988). A significant fraction of resting cellular energy or ATP turnover, from 1/3 for a typical cell to 2/3 for electrically-excitable cells (Alberts et al., 2002), fuels the Na\(^+\),K\(^-\)-ATPase. Generally cells with higher metabolic demand, such as epithelial cells involved in ion transport or liver cells with high rates of Na\(^+\)-coupled amino acid transport, have higher steady-state rates of passive Na\(^+\) entry. Less metabolically active cells, such as quiescent cultured fibroblasts or red blood cells, have much lower rates of steady-state Na\(^+\) entry (Lechene, 1988). In addition, the major routes of Na\(^+\) entry vary depending on the function of the cell. For example a large proportion (> 2/3) of the resting Na\(^+\) entry in renal proximal tubule cells is due to Na\(^+\) re-absorption via the Na/H exchanger (Harris et al., 1986).

The main purpose of this work is to elucidate major routes of steady-state Na\(^+\) entry in hair cells, with the hope of gaining a better understanding of both the function of the hair cell as a sensory transducer and the metabolic demands associated with this function.
Importance of $\text{Na}^+$ ion homeostasis to hair cell function in vivo

The electrophysiology of various hair cell types has been extensively studied (Ashmore, 1991; Guth et al., 1998). Yet little is known about $\text{Na}^+$ homeostasis in hair cells. Studies to date have established that isolated mammalian hair cells contain $\text{Na}^+$/H$^+$ and $\text{Na}^+$/Ca$^{2+}$ exchanger activity (Ikeda et al., 1992a; Ikeda et al., 1994; Chabbert et al., 1995; Ikeda et al., 1996); the intracellular [Na$^+$], however, was quite elevated (> 85 mM) in these studies. In addition, the activity of these exchangers was only shown for large perturbations. Thus we do not know from these studies what are the physiological steady-state rates of ion transport. Mroz et al (1993b) developed a technique to isolate goldfish hair cells with low intracellular Na$^+$ concentration. They found that the total steady-state Na$^+$ influx was surprisingly high. However, these studies did not determine the pathways through which the sodium enters.

Do transduction currents pose a sodium load to hair cells?

A portion of the cation influx into hair cells via transduction channels, either at rest or in response to stimulation, is carried by Na$^+$ ions. The steady influx of cations into hair cells due to the silent current must be countered by either passive or active efflux. Since transduction channels are non-selective among small cations (Corey and Hudspeth, 1979), either K$^+$, Na$^+$ or Ca$^{2+}$ ions may enter. Mammalian endolymph contains high K$^+$, low Na$^+$ (Table 1) and high free Ca$^{2+}$ concentrations relative to the inside of the cell, as well as a high positive potential of +80 mV relative to perilymph. Teleost fish have a lower positive endolymphatic potential (EP) than mammals (+ 5 to +10 mV), but they
may also have a significantly higher endolymphatic Na⁺ (Enger, 1964; Zuo et al., 1995; Payan et al., 1999). All hair cells have a driving force for net Na⁺ entry via transduction channels, unless the ratio of intracellular Na⁺ to endolymphatic Na⁺ concentrations ([Na⁺]/[Na⁺]ₑ) > 300 (mammal) or 20 (fish – based on values from Table 1 for EP and Vₘ). Thus some proportion of the resting silent current is carried by Na⁺ ions, which must be actively extruded via the Na⁺,K⁺-ATPase. The Na⁺ load could increase up to 5x the resting value if we assume that the average open probability of the mechanotransduction channels increases from 10% at rest to 50% during maximal sound stimulation.

We can estimate the Na⁺ load due to the silent current using in vivo measurements of standing currents in the cochlea. Zidanic and Brownell (1990) estimated the resting silent current to be ~500 pA per hair cell in the guinea pig cochlea. The portion carried by Na⁺ ions is proportional to the concentration ratio of Na⁺ to K⁺ in endolymph (Table 1), which in mammalian cochlea is 1/158, or .006 (Salt and Thalmann, 1988). The ratio of Na⁺ permeability to K⁺ permeability of a transduction channel is 0.9 (Corey and Hudspeth, 1979). Thus the sodium current into hair cells, absent stimulation, would be 0.54% of the total current, or 2.7 pA (entry rate of Na⁺ of 1.3 mM/min). During maximal stimulation the Na⁺ current could increase to 13.5 pA (i.e. factor of 5 increase). Since fish endolymph may have a higher Na⁺ concentration, we would expect the Na⁺ current via transduction channels to be even higher, even taking into account the lower endolymphatic potential. Assuming a conservative estimate of the endolymphatic [Na⁺] in the fish saccule (Table 1), the silent Na⁺ current would be 23 pA, which corresponds to a Na⁺ entry rate of 11.5 mM/min. In comparison, the total steady-state entry of Na⁺ in
goldfish hair cells was equivalent to a current of ~8 pA (Mroz et al., 1993a). Thus the silent current could actually be a substantial portion of the total Na⁺ entry.

<table>
<thead>
<tr>
<th></th>
<th>Mammal</th>
<th>Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺ current via transduction channels due to silent current (pA)</td>
<td>2.7</td>
<td>23.0</td>
</tr>
<tr>
<td>Na⁺ entry rate due to silent current (mM/min)</td>
<td>1.3</td>
<td>11.5</td>
</tr>
<tr>
<td>Na⁺ entry rate during maximal stimulation (5x the silent value) (mM/min)</td>
<td>6.5</td>
<td>57.5</td>
</tr>
<tr>
<td>Assumptions about ionic compositions of solutions</td>
<td>[Na⁺]_{endo} = 1 mM</td>
<td>[Na⁺]_{endo} = 15 mM</td>
</tr>
<tr>
<td></td>
<td>[K⁺]_{endo} = 158 mM</td>
<td>[K⁺]_{endo} = 144 mM</td>
</tr>
<tr>
<td></td>
<td>[Na⁺]_{per} = 145 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[K⁺]_{per} = 5 mM</td>
<td>Assume same as mammalian vestibular system¹</td>
</tr>
<tr>
<td>(Salt and Thalmann, 1988)</td>
<td>EP = 80 mV</td>
<td>EP = 10 mV</td>
</tr>
<tr>
<td></td>
<td>V₉₉ = 70 mV</td>
<td>V₉₉ = 13 mV</td>
</tr>
</tbody>
</table>

**TABLE 1.** Na⁺ currents or entry rates due to silent and maximal transduction currents in mammalian and fish hair cells. We make the following assumptions: [Na⁺]₉₉ = 15 mM, [K⁺]₉₉ = 120 mM, V₉₉ = -70 mV, cell volume = 1.2 pL, the total silent current into a hair cell is 500 pA (Zidanic and Brownell, 1990), the Na⁺ component of the silent current depends on the relative concentrations of [Na⁺]_{endo}/[K⁺]_{endo} in the endolymph, as well as the driving force → (EP-V₉₉) – V₉₉.

The Na⁺,K⁺-pump in most cells typically works at 1/3 of its physiologically-achievable maximal rate. During the short term the pump would only be able to keep up with an increase of Na⁺ entry rate by a factor of 3. Thus even in hair cells facing a low

¹ Measurements of Na⁺ concentration in fish saccule by other investigators are actually much higher than this value, but there may have been contamination of endolymph with perilymph, therefore we took a conservative value based on measurements in the mammalian vestibular system, as a lower bound for the endolymphatic [Na⁺] in fish.
sodium concentration apically, the small but also variable Na\(^+\) current via the transduction channels could pose significant demands on Na\(^+\) homeostasis in the cell.

**The apical ion load may overwhelm hair cells under certain pathophysiological circumstances.**

There are several pathophysiological situations during which the concentration of Na\(^+\) in the endolymph might increase. Vassout (1984) measured a reversible decrease in K\(^-\) concentration in the endolymph of 65 mEq/L after 1 hour of acoustic trauma (1.8 kHz, 105 dB SPL tone) in guinea pigs. To match the physical constraints of charge and osmotic balance, the decrease in K\(^-\) of 65 mEq/L must be offset by a concomitant increase in the endolymphatic [Na\(^+\)].

Apical Na\(^+\) could also significantly increase if the membranes separating the endolymph from the perilymph were to rupture. This has been hypothesized to occur during acute attacks of Ménière's disease (Schuknecht and Igarashi, 1986; Derebery, 1996). The volume of perilymph in a guinea-pig is 16 µL whereas the endolymphatic compartment is only 2 µL (Salt and Thalmann, 1988). Given the concentrations of Na\(^+\) and K\(^-\) in endolymph and perilymph (Table 1), mixing these two solutions produces concentrations (in both compartments) of [Na\(^-\)] = 129 mM and [K\(^+\)] = 22 mM. This large Na\(^+\) concentration apically could overwhelm Na\(^+\) homeostasis in hair cells. In addition the elevated K\(^+\) concentration basolaterally might cause excitotoxic damage to hair cells or to afferent terminals of the auditory nerve fibers. The results reported in this thesis allow us to predict the consequences for hair cells of mixing these two fluids.

Bosher (1979) measured the effects of anoxia on cation concentrations and pH in the endolymph in both guinea-pig and rat. Anoxia caused an immediate increase in Na\(^+\)
concentration at a rate of 0.63 mM/min, and this rate tripled after 15 minutes. Endolymphatic pH decreased from 7.4 to 7.1 within the first 10 minutes. Both the pH decrease and Na\(^+\) increase might overload the ion-homeostatic mechanisms of hair cells.

In an isolated preparation of the gerbil organ of Corti, Liu and Siegel (1996) found that exposure of the endolymphatic compartment to high-Na\(^+\) artificial perilymph caused hair-cell sodium and calcium concentrations (measured with the dyes Sodium Green and fluo3) to increase over time. In isolated mammalian hair cells, which face a high Na\(^+\) medium, resting intracellular Na\(^+\) concentrations are elevated (Ikeda et al., 1992a; Chabbert et al., 1995). Mroz and colleagues (1993b) found that isolation of goldfish hair cells using a procedure which breaks a large number of tip links, thereby closing off transduction channels (Assad et al., 1991; Crawford et al., 1991), yields isolated cells with physiologically reasonable intracellular Na\(^+\) concentrations of less than 20 mM. Thus transduction channels are a potentially significant pathway for Na\(^+\) entry which could overload the capability of the Na\(^+\),K\(^+\)-ATPase in cases where the apical Na\(^+\) concentration increases.

**There may also be a substantial basolateral Na\(^+\) load on hair cells.**

In addition to apical ion entry, Na\(^+\) enters hair cells via ion channels or Na\(^+\)-coupled transporters in the basolateral membrane, which faces the high-Na\(^+\) perilymph. One example is the Na\(^+\)/Ca\(^{2+}\) exchanger, which extrudes Ca\(^{2+}\) ions using the downhill entry of Na\(^+\) ions (Ikeda et al., 1992a). Hair cells are known to have high spontaneous rates of steady-state neurotransmitter release (Furukawa and Ishii, 1967), which is
activated by Ca$^{2+}$ entry through voltage-gated calcium channels. There may also be concomitant Na$^+$ entry to relieve such a calcium load via the Na$^+/Ca^{2+}$ exchanger.

Na$^+$-coupled uptake of substrates need for synthesis of neurotransmitter is another potentially significant basolateral entry pathway. Ryan and Schwartz (1984) measured uptake of amino acids in the cochlea and found that glutamine was preferentially taken up by inner hair cells. This preferential labeling may be due to the use of glutamine as a substrate for the neurotransmitter at the hair cell afferent synapse. Glutamine can also be an important substrate for energy metabolism in tissues which are poorly vascularized, like the Organ of Corti (i.e. the sensory epithelium of the cochlea). Glutamine transport is often coupled to Na$^+$ entry (Broer and Brookes, 2001). Na$^+$-coupled uptake of other amino acids may also occur in hair cells as it does in other cell types (Cohen and Lechene, 1990).

Thus any increase in general metabolic demand, such as might occur during acoustic stimulation, would be expected to cause an increase in basolateral Na$^+$ entry in addition to any increases in the apical load via transduction channels.

**Goldfish hair cells as a model system**

Goldfish hair cells have a very high Na$^+$ turnover (Mroz et al., 1993a), and large numbers can be isolated with low intracellular [Na$^+$], (Mroz et al., 1993b). Thus these cells are an ideal system for studying Na$^+$ ion homeostasis. They also are phylogenetically quite old (Popper and Coombs, 1980), and hence represent a generic type of hair cell, more similar to mammalian inner hair cells than outer hair cells. The
following two sections outline additional reasons for examining goldfish hair cells in particular.

**The electrophysiology of goldfish hair cells has been extensively studied.**

There have been many studies performed on both the anatomy and the physiology of goldfish hair cells. For this thesis, hair cells were isolated from three organs of the goldfish inner ear, the saccule, lagena, and utricle. Von Frisch (1936) established that the major organs of hearing in bony fish are the saccule and lagena. The utricle and semi-circular canals are involved in balance. Goldfish are part of a group of bony fish called Ostariophysii, which have better hearing sensitivity than many other fishes. In these kinds of fish, sound pressure variations are picked up by the air bladder located in the body and are conducted to the saccule and lagena by a chain of three Weberian ossicles.

The first electrophysiological responses of an Ostariophysian fish to sound were measured by Furukawa and Ishii (1967) in goldfish. They established two different populations of afferent nerve fibers. S1 fibers from the rostral end of the saccule had large diameters and no spontaneous activity, and S2 fibers from the caudal end had small diameters and displayed spontaneous discharges of between 10 and 200 spikes/s. Sugihara and Furukawa (1989) subsequently established that there are two distinct types of hair cells based on morphology and responses to voltage and current clamp, which were also differentially innervated by these fibers. Figure 2 lists the anatomical and physiological differences between these two types of cells.

Rostral cells were very short and ovoid-shaped (13-20 μm in length) and showed a damped oscillatory voltage response to depolarizing currents. Cells in the caudal end were more variable in shape with both short (< 20 μm in length) and tall hair cells (25 –
50 μm in length). Tall cylindrical-, eggplant-, or gourd-shaped cells gave large, spike-type voltage responses to depolarizing current pulses (above a certain threshold). The caudal end also contained some damped, oscillatory type cells, but they were all short (width over 1/3 of length). Thus morphologically, spike-type cells are easily distinguished from oscillatory cells. Tall and short hair cells were also found in the lagena and utricle, with similar physiological responses to the same cell types from the saccule.

Currents in the spike-type (tall) hair cells were dominated by Na⁺ currents. In voltage-clamp experiments, the large surge of inward current due to a voltage step was completely abolished either by 30 μM of the Na⁺-channel blocker TTX or by replacement of Na⁺ in the medium with choline. Short hair cells exhibited an inwardly rectifying I₉⁻ like current for hyperpolarizing steps which can carry Na⁺ ions.

Generally speaking the tall hair cells far outnumber the short ones after isolation of cells from the entire saccule, possibly because of loss of the short-ovoidal cells from the rostral end during the procedure (Sugihara and Furukawa, 1989; Mroz et al., 1993b). Thus our data come from tall spike-type hair cells. Several of the properties of tall hair cells (Figure 2), such as TTX-sensitive Na⁺ currents and Ca²⁺ currents involved in spike generation, spontaneous transmitter release (absent stimulation), little calcium binding protein in the cell body, and large synaptic bodies, may be particularly relevant for Na⁺ ion homeostasis.
**Figure 2.** Anatomical and physiological differences between two hair cell types in the goldfish saccule (reviewed in (Lanford et al., 2000)). This figure shows a drawing of the shape of the saccule and the two major cell types found at either end (rostral versus caudal). The stippled area on the rostral end is meant to show the area innervated by the large S1 fibers (Sugihara and Furukawa, 1989). References: ¹ (Sugihara and Furukawa, 1989), ² (Furukawa and Ishii, 1967), ³ (Saidel et al., 1995) ⁴ (Lanford et al., 2000), ⁵ (Sugihara and Furukawa, 1996).

<table>
<thead>
<tr>
<th>Rostral:</th>
<th>Caudal:</th>
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<tr>
<td>- Short (&lt; 20 μm) eggplant or pear shape⁴</td>
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<td>- Small amplitude damped oscillation to depolarizing currents⁴</td>
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<tr>
<td>- Innervated by large diameter S1 fibers²</td>
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<td>- S1 fibers do not have spontaneous activity²</td>
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<tr>
<td>- Best frequencies 790-1770 Hz²</td>
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<td>- Small synaptic bodies³,⁴</td>
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<tr>
<td>- Calcium binding protein S-100 throughout cell body³</td>
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<tr>
<td>- Mitochondria located above and below nucleus³,⁴</td>
<td></td>
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<td>- Currents during voltage clamp¹:</td>
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<tr>
<td>- Ca²⁺-activated K⁺ current</td>
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<td>- Outward rectifying A current</td>
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<td>- Iₜ</td>
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<td>- Vm rest¹ = −70mV</td>
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<td>- Tall (&gt;25μm) cylindrical or gourd-shaped⁴</td>
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<tr>
<td>- Spike-type responses to depolarizing currents⁴</td>
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<tr>
<td>- Innervated by small diameter S2 fibers²</td>
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<tr>
<td>- S2 fibers have spontaneous rates up to 200/s²</td>
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<tr>
<td>- Best frequencies 120-290 Hz²</td>
<td></td>
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<tr>
<td>- Large synaptic bodies³,⁴</td>
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<tr>
<td>- Calcium binding protein S-100 only at apical surface³.</td>
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<tr>
<td>- Mitochondria mostly sub-nuclear³,⁴</td>
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<tr>
<td>- Currents during voltage clamp¹:</td>
<td></td>
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<tr>
<td>- TTX-sensitive Na⁺ currents</td>
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<tr>
<td>- Ca²⁺ currents</td>
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<tr>
<td>- Inward rectifying K⁺ channels that set the large negative resting potential⁵</td>
<td></td>
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<tr>
<td>- Vm rest¹ = −90 to −100 mV</td>
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Different hair-cell types vary in their ability to deal with Na\(^+\) loads.

Classic experiments showed that mammalian and fish hair cells, in vivo, respond quite differently to disruption of the normal K\(^+\)/Na\(^+\) concentrations in the endolymphatic compartment. Konishi (1966) found that perfusion of perilymph into the endolymphatic compartment of guinea pig caused the cochlear microphonic\(^2\) to decrease by 50% within the first ten minutes, before there were any significant changes in the endolymphatic potential. Interestingly, Matsuura et al. (1968) perfused high Na\(^+\) into the endolymphatic compartment of goldfish saccule and measured only a slight effect on the cochlear microphonic response. Thus is appears that hair cells in the fish are better able to deal with an apical sodium load than those from mammal.

Matsuura and colleagues suggested that the differences in vulnerability to apical Na\(^+\) between the guinea-pig and fish might have to do with fish having almost equal portions of Na\(^+\) and K\(^+\) in the endolymph (Enger, 1964). This idea has also been suggested by other studies on fish endolymph composition (Payan et al., 1997; Payan et al., 1999), but remains controversial as it can be difficult to sample the endolymph without causing contamination by perilymph. However, even assuming a lower bound on fish endolymph Na\(^+\) concentration of 15 mM, based on the mammalian vestibular system (Smith et al., 1954), there would be a substantial apical Na\(^+\) load via transduction channels. Hence fish hair cells may be pre-adapted to deal with large apical Na\(^+\) loads by having higher (basolateral) Na\(^+\),K\(^+\)-ATPase activity.

\(^2\) The cochlear microphonic is a summed extracellular voltage response to sound stimulation measured with an electrode inserted into the inner ear. Thus the CM is a measure of hair cell activity.
Another interesting piece of evidence suggests that hair cells of mammalian otolithic vestibular organs (i.e. saccule and utricle) may be better equipped to deal with apical Na$^+$ loads than are mammalian cochlear hair cells. A mutation of the mouse *Isk* gene, which is a potassium channel involved in K$^+$ secretion into the endolymph by strial-marginal cells in the cochlea and by vestibular dark cells in the vestibular system, causes differing amounts of hair cell degeneration depending on the organ (Vetter et al., 1996). Hair cells in the cochlea and semi-circular canal organs degenerate by P3 and P10 respectively, whereas saccular and utricular hair cells appear to be "hardier" since they do not degenerate for months. Since the mutation causes loss of K$^+$ secretion, it might lead to a low K$^+$ and high Na$^+$ concentration in the endolymphatic compartment. Perhaps the hair cells of the utricle and saccule are better equipped to deal with the resulting apical sodium load.

There may also be differences in expression of the Na$^+$,K$^+$-ATPase among hair cells in cochlear and vestibular tissues. An immunohistochemical study (ten Cate et al., 1994) used antibodies to stain for subunits α1, α2, α3, and β1 and β2 of the Na$^+$,K$^+$-ATPase in rat cochlear and vestibular tissues. They found that α3β1 was expressed at the bases of inner hair cells, as well as in the sensory and supporting cells of the crista$^3$ and utricular and saccular maculae$^4$. There was almost no staining of supporting cells in the cochlea. There was, at most, a slight staining at the bases of outer hair cells (OHCs) for α2. Thus it appears that inner hair cells of the cochlea, as well as hair cells and supporting cells in the vestibular epithelia, express Na$^+$,K$^+$-ATPase. OHC staining was

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$^3$ The crista is the sensory epithelium of the semi-circular canals, the organs of the vestibular system which detect angular accelerations due to head movements.

$^4$ The maculae are the sensory epithelia of the utricle and saccule. In the mammals, these organs of the vestibular system which detect gravity and linear acceleration, respectively. In the fish the saccule is a hearing organ.
very weak in comparison and indicates either a low activity of Na\textsuperscript{+},K\textsuperscript{+}-ATPase, or that the OHC may express other undiscovered isoforms. If OHCs do have low Na\textsuperscript{+},K\textsuperscript{+}-ATPase, it may be that they have sacrificed some ion-homeostatic capability in order to carry out their function as electrical-mechanical transducers. Thus the packing of the "motor protein" prestin (Zheng et al., 2000) in the lateral membranes of OHCs may be at the expense of Na\textsuperscript{+},K\textsuperscript{+}-ATPase.

In the mammalian ear, the cells in the lateral wall tissues involved in recirculation of K\textsuperscript{+} ions (strial marginal cells in the cochlea, dark cells in the vestibular system) stained much more intensely for the Na\textsuperscript{+},K\textsuperscript{+}-ATPase than did the sensory epithelia (Schulte and Adams, 1989; Spicer et al., 1990). Burnham and Stirling performed a quantitative study to measure ouabain binding sites (i.e. Na\textsuperscript{+},K\textsuperscript{+}-ATPase) in bullfrog saccule. They found that the density of ouabain binding was as high in hair cells as it was in the nerve fibers, with a value of 3000 per μm\textsuperscript{2} (Burnham and Stirling, 1984a). Interestingly, in the dark cells of the saccule, the density of ouabain binding sites was 4500 per μm\textsuperscript{2} (Burnham and Stirling, 1984b). Thus, at least in some non-mammalian vertebrates, hair cells have a high expression of Na\textsuperscript{+},K\textsuperscript{+}-ATPase. We know what the function of a high expression of this enzyme is in the dark cells: K\textsuperscript{+} recirculation. Based on the findings reported in this thesis, we now have a better understanding of the importance of high Na\textsuperscript{+}-pump activity in hair cells.
CHAPTER 3: Sodium entry pathways in isolated goldfish hair cells

ABSTRACT

Hair cells are the mechanosensory transducers of the inner ear and lateral line organ. While the electrophysiology of hair cells has been extensively studied, little is known about the vital background processes that maintain the steady-state intracellular ionic environment. Therefore, we characterized the steady-state Na\(^+\) influx and efflux pathways in isolated goldfish hair cells. Intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)) and pH were measured using the fluorescent probes sodium-binding benzofuran isophthalate (SBFI) and 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF), respectively. The steady-state [Na\(^+\)]\(_i\) of 12.8 mM and pH of 7.4 were similar to values from many other cell types. The steady-state Na\(^+\) entry was determined by measuring the initial rate of increase in [Na\(^+\)]\(_i\), during inhibition of the Na\(^+\),K\(^+\)-ATPase (Na\(^+\)-pump), and found to be 9 mM/min. The steady-state Na\(^+\) influx was estimated to be 25 pmol/cm\(^2\)/s, which is as fast as that measured in the squid axon (Hodgkin and Keynes, 1955) and an order of magnitude faster than mammalian cardiac myocytes (Despa et al., 2002). The maximal Na\(^+\) extrusion rate of the Na\(^+\),K\(^+\)-ATPase, estimated from the initial rate of decrease in intracellular Na\(^+\) concentration after release from pump inhibition, was double the steady-state entry rate. Based on this maximal rate, we estimate that there are approximately 1000 pump sites per \(\mu m^2\) of cell membrane. We sought to uncover the entry pathways for such a high steady-state Na\(^+\) influx. Pharmacological agents and
manipulations of extracellular composition were used to dissect apart the contributions of various transporters or channels, including transduction channels, Na⁺/Ca²⁺ exchangers, Na⁺/H⁺ exchangers, and TTX-sensitive Na⁺ channels. We found that 1/3 of the total steady-state Na⁺ entry flowed through the few transduction channels that remained intact despite exposure to a Zero-Calcium EGTA solution during the dissociation. 1/4 appeared to enter via the Na⁺/Ca²⁺ exchanger. Consistent with a significant component of steady-state Na⁺ entry via the Na⁺/Ca²⁺ exchanger, we found that nifedipine, a blocker of L-type calcium channels, also reduced the Na⁺ entry rate. Another 1/5 of Na⁺ entry was via the Na⁺/H⁺ exchanger. Thus three major pathways can account for ¾ of the total steady-state Na⁺ entry in goldfish hair cells. In addition, K⁺-depolarization reduced both [Na⁺], and steady-state Na⁺ entry, suggesting that there is either another ion channel component of steady-state Na⁺ entry, or that the Na⁺/Ca²⁺ exchanger is K⁺-dependent, as in rod photoreceptors. Although hair cells were traditionally described as passive resistors, these results demonstrate that goldfish hair cells have a significant metabolic load arising from demands on these Na⁺-ion transport processes.
INTRODUCTION

Most animal cells maintain a large electro-chemical gradient for Na$^+$ ions across the plasma membrane. A third or more of a cell’s steady-state ATP turnover is used by the Na$^{+}$,K$^{+}$-ATPase (Skou, 1989), to maintain a low intracellular Na$^+$ concentration ([Na$^+$])$_i$ (Clausen et al., 1991; Rolfe and Brown, 1997; Alberts et al., 2002). Na$^+$ influx via symporters or antiporters energizes processes that regulate intracellular pH and ion content and that exchange nutrients and metabolites with extracellular fluids. Na$^+$ influx via voltage-gated ion channels is the basis of action potential generation in excitable cells. Cells with high metabolic demands generally have high steady-state Na$^+$ influx balanced by the Na$^{+}$,K$^{+}$-ATPase (Na$^+$-pump). For example, Na$^+$ influx in renal proximal tubule cells is over two orders of magnitude higher than in red blood cells (Lechene, 1988).

Hair cells are the primary sensory cells of auditory and vestibular organs. Hair bundles on the apical membranes of hair cells are made up of individual stereocilia, which contain mechanosensitive ion channels. Mechanical stimulation increases the number of channels that are open. However, even absent stimulation, 5-15% of these channels are open at rest (Hudspeth and Corey, 1977; Ricci and Fettiplace, 1998), allowing a “silent current” into the hair cell (Zidanic and Brownell, 1990).

The apical extracellular surfaces of a hair cells face a unique high-K$^+$ fluid, the endolymph. Since the transduction channels are cation-selective but otherwise nonspecific among small cations (Corey and Hudspeth, 1979), the predominance of K$^+$ in the endolymph means that most of the cation current passing inward through transduction
channels is carried by $K^+$ ions. The extracellular surfaces of the basolateral membranes of hair cells face a fluid, called perilymph, with the high $Na^+$ and low $K^+$ characteristic of most animal extracellular fluids. Across the basolateral membranes, the electrochemical potential difference for $K^+$ favors exit of that ion. Thus the $K^+$ fluxes pose no energetic demands on hair cells.

$K^+$ ions are actively recirculated from perilymph back to endolymph by cells in the epithelium located laterally to the hair cells, completing the circuit of the “silent current” (Davis, 1968; Zidanic and Brownell, 1990; Kikuchi et al., 2000). Since $K^+$ ions are the major carrier of the currents associated with transduction, hair cells have traditionally been described as variable resistors with only minimal metabolic demands (Davis, 1968).

The discovery of extremely rapid sodium turnover in sensory hair cells of the goldfish (Mroz et al., 1993a) thus poses a challenge to our understanding of these cells’ metabolic demands. Hair cells from goldfish were found to have $Na^+$ turnover rates as fast as those of mammalian epithelial cells engaged in rapid ion transport, and orders of magnitude faster than those of other cell types in several species (Mroz et al., 1993a). Goldfish hair cells are an ideal model for studying $Na^+$ ion balance, both because they have rapid $Na^+$ turnover, and because large numbers of healthy cells can be isolated with low steady-state $[Na^+]_i$ (Mroz et al., 1993b). In addition, the electrophysiology of isolated goldfish hair cells has been extensively characterized with the whole-cell patch clamp method (Sugihara and Furukawa, 1989, 1996).

To discover why goldfish hair cells have such a high $Na^+$ turnover rate, we applied the technique of fluorescence ratio ion imaging to investigate major pathways of
steady-state $\text{Na}^+$ entry. Almost all of the $\text{Na}^+$ entry could be accounted for by a combination of the following pathways: transduction channels left open after the cell dissociation procedure, the $\text{Na}/\text{Ca}^{2+}$ exchanger, the $\text{Na}^+/\text{H}^+$ exchanger, and a component sensitive to $\text{K}^+$-depolarization.

The remarkably high steady-state $\text{Na}^+$ turnover in goldfish hair cells may explain why, in classic in vivo experiments, they were much less vulnerable to perfusion of a high $\text{Na}^+$ concentration into the endolymphatic compartment than were mammalian cochlear hair cells (Konishi et al., 1966; Matsuura et al., 1971).
METHODS

Solutions and chemicals

Unless otherwise noted, all solutions and chemicals were purchased from Sigma-Aldrich, Inc. The compositions of all perfusion solutions, including the Standard control solution, are listed in table 1.

The solutions used for cell dissociation were made as follows: MEM Hepes (M2645) was adjusted to an osmolality of 256 mOsm/kgH_2O by dissolving the powder for the 1 liter formulation into a final volume of 1.5 liters, buffering with Hepes/NaOH (3.3 mM Hepes, ~10 mM NaOH), and adding an extra 45 mM NaCl. HBSS-M (H2387-Zero-Ca\(^{2+}\), Zero-Mg\(^{2+}\)) was adjusted to an osmolality of 256 mOsm/kgH_2O by buffering with Hepes/NaOH (10mM Hepes, ~5mM NaOH) and diluting the final solution by 10% (in the petri dishes on the day of experiment). Both MEM and HBSS-M were adjusted to a pH of 7.5 with NaOH. Collagenase D (Boehringer Manheim #1 088 858), Dispase (GibcoRL #17105-032), and Bovine Serum Albumin (Sigma BSA, fatty-acid free) were prepared as 12.5 and 50 mg/ml aliquots and stored at −20°C.

Drugs

All stock solutions were stored in the dark. Amiloride was made as a 0.5 M stock solution in DMSO and stored at −20°C. Bumetanide and ouabain were made as 100 mM stock solutions in DMSO and stored at room temperature. Ethylisopropylamiloride (EIPA) and nifedipine were made as 100 mM stock solutions in DMSO and stored at 8°C. Dihydrostreptomycin was dissolved as a 150 mM stock in
deionized H₂O, and stored in a refrigerator for up to 1 week. Nigericin was made as a 14 mM stock in ethanol, and gramicidin was made as a 5mM stock in DMSO; both of these were stored at −20°C. TTX was made as a 1mM stock in deionized H₂O and stored as 25-µl aliquots at −20°C. Carbenoxolone was made as a 100 mM stock in deionized H₂O and stored at 8°C. BCECF-AM and SBFI-AM (Molecular Probes, Eugene, Oregon) were made as 2 mM stock solutions in DMSO (in their original 50-µg vials) and stored with desiccant at −20°C. Pluronic F-127 20% w/v (Molecular Probes, Eugene, Oregon) in DMSO was stored at room temperature. All drug stocks were dissolved in solutions at their final concentrations just before use.

**Hair cell dissociation**

Hair cells were prepared according to Protocol B from Mroz et al (1993b). Briefly, 8-18 cm long comet goldfish (*Carrasius auratus*) were chilled in water to near 0°C, then decapitated just behind the gills. The top of the head was removed and the overlying brain lifted away to expose a transparent bony capsule on the ventral side containing the lagena and saccule. Forceps were placed just inside the open holes in the bony capsule and the saccule and lagena removed from both sides. Often the utricle was also removed along with the lagena and saccule. Otherwise the utricle was taken from the wall of the head, lateral to the opening of the bony capsule. The tissues were placed into a 25-mm petri dish containing HBSS-M (adjusted to fish osmolarity, see above). Each tissue was cleaned of blood vessels, nerves, and otoliths, and the lumenal sides of the sensory macula exposed to the medium by opening up any overlying tissue. The cleaned tissues were then transferred into HBSS-M containing 1 mM EGTA/3 mM NaOH for 20 minutes, followed by enzyme treatment in HBSS-M containing 0.625
mg/ml Collagenase D and 2.5 mg/ml Dispase for 8 minutes. The tissues were then allowed to rest for 10 minutes in a petri dish containing 1.5 ml MEM supplemented with 0.5 mg/ml BSA. The cells were released by mincing the tissue. The MEM solution containing the isolated cells was gently tritured at a volume of 0.3 ml 30 times with a 1-ml wide-bore pipette tip on a Gilson Pipetman® (Rainin). Subsequently 450 µl of cells from the MEM petri dish were loaded with the fluorescent probe and the remaining cells in MEM were kept at 8°C for later use (up to about 6 hours).

*Loading of fluorescent ratio ion indicators*

Cells were loaded with the membrane-permeant acetoxyethyl (AM) ester form either of the Na⁺ indicator SBFI or of the pH indicator BCECF. For SBFI, equal volumes of SBFI in DMSO (from a 2mM stock) and Pluronic F-127, a dispersing agent, were dissolved in MEM at a concentration of 100 µM and then mixed with 450 µl of cells in MEM inside a 1.5 ml eppendorf tube to give a final concentration of 10 µM SBFI (1% DMSO and Pluronic F-127). For BCECF, the final concentration was 1-2 µM (0.1 % DMSO). The cells were then transferred to a coverglass-bottomed, open, laminar-flow, perfusion chamber (Warner instruments, Hamden, CT). The coverglass was pretreated with a 50 µl drop of 1 mg/ml concanavalin A (Sigma C2010) at 37°C for 1 hour and extensively washed with deionized water and MEM. The perfusion chamber was placed in a humidified petri dish and the cells were allowed to load with the fluorescent probe at room temperature in the dark. For SBFI the loading time was 60 minutes and for BCECF the loading time was 30 minutes.
Measurement of pH or [Na⁺]

The perfusion chamber was mounted on the stage of an inverted Zeiss 405M epi-fluorescence microscope equipped with phase and differential interference contrast optics. A dual-head peristaltic pump, running at a speed of 500 µl/min, controlled perfusion of solution into and out of the chamber. All experiments were carried out at room temperature. The cells were perfused with the Standard solution (Table 1) for 20 minutes to wash away the dye and allow continued hydrolysis of any AM ester left inside the cells.

ISee analytical image processing software for Linux (Isee Imaging Systems, Raleigh, NC) was used to control the excitation filter wheel, shutters, emission filter wheel, and the CCD camera. The dual excitation filter wheel (Ludl Electronics Products, Ltd., Hawthorne, NY) contained a wheel with bandpass filters (bandwidths of 25 nm), and another wheel with neutral density filters, in addition to a shutter. A beam of light from a 100-watt Mercury Arc lamp with a quartz condenser lens passed through a heat filter and the dual-excitation filter wheel before entering the rear port of the microscope and the filter cube inside. For SBFI, the filter cube contained a 400-nm long pass dichroic mirror. For BCECF, the filter cube contained a wide band excitation filter from 450-490 nm, a 510-nm dichroic mirror, and a bandpass emission filter at 535 nm (25 nm bandwidth). The excitations wavelengths for each dye were: SBFI, 340 nm (Na⁺-insensitive wavelength) and 380 nm (Na⁺-sensitive), BCECF, 450 nM (pH insensitive) and 490 nM (pH sensitive). A 20x/0.75 NA Nikon Fluar objective lens was used for both phase contrast and epi-fluorescence imaging. For SBFI, the fluorescence emission from the cells was passed through a 523-nm bandpass filter (40-nm bandwidth) in the emission
wheel. Emissions were captured by the CCD camera (liquid-cooled Photometrics CH350) and stored in an image list on the hard drive. Each pair of images was collected once every 30 or 60 seconds (~5 s between each excitation wavelength). Both the neutral density filter and the exposure time of the excitation were adjusted to minimize the intensity of light exposure and to give an emission at least 3-6 times the background signal. The ratio of the emission from the two different wavelengths (i.e. 340/380 for SBFI, 490/450 for BCECF) in a small region of interest (ROI) inside the cell was monitored online and the individual background-subtracted and ratio values (the background was taken as a small ROI adjacent to the cell) were also saved in a table to be analyzed later in Excel.

*Calibration*

At the end of each experiment, an in situ calibration was performed for either intracellular Na\(^+\) concentration or pH. During calibration, a separate perfusion line was used in order to avoid contaminating the regular perfusion lines with ionophores (Bevensee et al., 1999). A manifold (Warner instruments, Hamden, CT) was used to select between the regular solution line (from the peristaltic pump) and the calibration solution line (gravity fed), and the small (1.3 cm) piece of tubing between the manifold and perfusion chamber was replaced after each calibration.

**SBFI/Na\(^+\) calibration.** The pore-forming antibiotic gramicidin was perfused (5 μM) along with 3-5 solutions of differing Na\(^+\) concentrations. These calibration solutions were made by combing different volumes of equimolar high K\(^+\)/low Na\(^+\) and high Na\(^+\)/zero-K\(^+\) solutions; Cl\(^-\) was replaced by gluconate to provide a concentration close to [Cl\(^-\)].
(see Table 1 for recipe). A range of extracellular Na\(^+\) concentration from 2 mM up to 100 mM was used for the calibration. In most cases, a linear regression between the fluorescence ratio and each extracellular Na\(^+\) concentration provided a reliable calibration curve. Occasionally a non-linear hyperbolic fit was required, especially at higher Na\(^+\) concentrations (i.e. > 60 mM), where the curve of ratio versus [Na\(^+\)]\(_i\) can start to saturate. In each cell, we performed at least a 3-point Na\(^+\) calibration at Na\(^+\) concentrations up to 60 mM.

Figure 1 shows an example of a typical calibration, from 2 to 100 mM Na\(^+\). In this case the relationship between ratio and Na\(^+\) concentration was linear. The emission from the 340 nM excitation wavelength was quite insensitive to changes in intracellular Na\(^+\) concentration, and hence was used to monitor whether there were large changes in the state of the cell. A large change in emission in response to 340 nm illumination was taken as an indicator of major cell volume change or of cell membrane rupture and those cells were subsequently excluded from analysis.

**BCECF/pH calibration.** The ionophore nigericin (10 \(\mu\)M) along with a high K\(^+\)-gluconate solution was used to clamp intracellular pH to extracellular pH. 3 different pH solutions were used in the range 6.5 to 7.8 with the nigericin only in the solution of mid-range pH (around 7.3), which was perfused first. A linear fit to the average ratio at each pH was used to produce calibration curves.

**pH Buffering capacity**

We measured the intracellular pH buffering capacity by adding or removing a known acid or base load and measuring the change in intracellular pHi, using BCECF.
We used the ammonium chloride (NH₄Cl, 10 mM) technique according to Boron and De Weer (1976). Using the known pH₀ and the constant for dissociation of NH₄⁺ (K₀ = 1.86×10⁻⁵ moles/Liter), the extracellular concentration of NH₃ can be calculated. Assuming equilibrium of NH₃ across the cell membrane, [NH₄⁺], can then be calculated using the measured pHᵢ, either at the peak of the alkalinization (for NH₄⁺ addition), or from the pHᵢ just before the acidification (control, for NH₄⁺ withdrawal). The buffering capacity (βᵢ) is: βᵢ = [NH₄⁺]/ΔpHᵢ, where ΔpHᵢ is the change in pHᵢ, either from the control value to the peak value during the alkalinization, or from the control value to the minimum value during the acidification.

**Steady-state Na⁺ entry rate**

When the cell is in a steady-state, [Na⁺]ᵢ is constant because the passive influx of Na⁺ is balanced by active efflux via the Na⁺,K⁺-ATPase. We therefore measured the steady-state Na⁺ entry rate as the initial rate of increase in [Na⁺]ᵢ immediately after inhibition of the Na⁺,K⁺-ATPase (Abraham et al., 1985; Cohen and Lechene, 1989; Mroz et al., 1993a; Rose and Ransom, 1997a; Despa et al., 2002). We reversibly inhibited the pump by perfusion of the normal Standard solution without potassium ion (Zero-K⁺ solution – see Table 1). We performed a linear regression to the initial values of [Na⁺]ᵢ versus time after inhibition of the pump. The slope of this line (i.e. d[Na⁺]ᵢ/dt) gives the initial change in Na⁺ versus time in units of mM/min (mmoles/(Liter*time)).

Units of mM/min are used throughout rather than the traditional flux units of mol/(area*time). During Na⁺-pump inhibition, no gross changes in cell size or shape (i.e. volume) were detected, either visually or by monitoring the Na⁺ insensitive wavelength,
F340. Thus the change in concentration versus time reflects, for a constant cell volume and surface area, the net number of moles/s transported.

For a hair cell with a diameter of 7 μm and length of 30 μm, the volume is 1.2 pl and the surface area is 736 μm² (cylindrical geometry, excluding hair bundle). A 1mM/min Na⁺ entry rate thus corresponds to an influx of 2.7 pmol/cm²s, or a univalent ion current of 2 pA.

**Statistics**

Results are presented as means ± SEM (standard error of the mean) and statistical significance was tested using either paired (for repetitions within a cell) or unpaired Student’s t-tests (significance defined as p < 0.05). A one-way analysis of variance and multiple-range test (Dunnett) were also used to detect differences among multiple treatments, using the Analyze-IT statistical software.
RESULTS

Steady-state $[Na^+]_i$ and $Na^+$ turnover

Fluorescence ratio ion imaging in SBFI-loaded cells gave a steady-state $[Na^+]_i$ of 12.7 ± 0.8 mM (n = 107 cells). This is somewhat lower than reported in a previous study (Mroz et al., 1993b), which used electron-probe analysis to measure ion contents in isolated goldfish hair cells ($[Na^+]_i$ of 18.3 mM).

We removed $K^+$ from the extracellular solution (Zero-$K^+$) to inhibit the $Na^+,K^+$-ATPase and measured the initial rate of increase in $[Na^+]_i$, a measure of the steady-state $Na^+$ entry rate. Figure 2 shows a typical experiment in which two cells underwent two consecutive Zero-$K^+$ inhibition and recovery treatments (the second one inhibited for a longer time). For both cells, linear fits to the initial increase in $[Na^+]_i$ for the two treatments gave similar estimates of steady-state $Na^+$ entry rates (~12 mM/min). Even though the $[Na^+]_i$ during the second treatment reached a value of 80 mM, the cells were still able to rapidly recover back to the baseline level after perfusion of the $K^+$-containing standard solution. Typically cells were able to recover from at least 4 $Na^+$-pump inhibition treatments.

The average steady-state $Na^+$ entry rate was 9.0 ± 0.4 mM/min (n = 107). The $Na^+$ turnover time was 1.4 minutes (12.7 mM ÷ 9.0 mM/min). This rapid turnover time was similar to that measured in renal proximal tubule cells and faster than many other cell types (Lechene, 1988).

Removing $K^+$ from the extracellular solution is likely to hyperpolarize the membrane potential. Hyperpolarization in turn might increase the driving force for
sodium entry, causing an overestimate of the true steady-state entry rate of sodium. We therefore performed experiments to compare the entry rate of Na\(^+\) measured with Zero-K\(^+\) to that measured with 150 μM ouabain, a specific inhibitor of the Na\(^+\),K\(^+\)-ATPase. Blocking this electrogenic pump with ouabain is expected to have a slightly depolarizing effect on the membrane potential, opposite to the hyperpolarization from K\(^+\)-removal. Figure 3 shows a typical experiment, in which 4 cells were first exposed to Zero-K\(^+\) to inhibit the pump, and then allowed to recover. The cells were then perfused with 150 μM ouabain (with K\(^+\)). In a paired comparison, the steady-state Na\(^+\) entry rate measured with 150 μM ouabain was 1 ± 0.3 mM/min higher than for Zero-K\(^+\) (9.8 ± 0.8 mM/min with ouabain versus 8.8 ± 0.7 mM/min for Zero-K\(^+\), n = 10). Thus the reversible Zero-K\(^+\) method for inhibiting the Na\(^+\)-pump does not overestimate the steady-state sodium entry rate, and was generally used in preference to the poorly reversible ouabain treatment.

We also determined the maximal rate at which the Na\(^+\),K\(^+\)-ATPase can extrude Na\(^+\). The initial rate of decrease of intracellular Na\(^+\) concentration after release of pump inhibition (i.e. after return to control [K\(^+\)]\(_o\)) in Na\(^+\)-loaded cells is an estimate of the maximal Na\(^+\) extrusion rate of the Na\(^+\),K\(^+\)-ATPase (Lechene, 1988). When measured in intact cells, the Na\(^+\),K\(^+\)-ATPase rate saturates at [Na\(^+\)]\(_i\) > 30mM (Zahler et al., 1997; Despa et al., 2002). The average initial rate of decrease in [Na\(^+\)]\(_i\) in hair cells Na\(^+\)-loaded to > 30 mM was 16.2 ± 0.8 mM/min (n=48). This rate of decrease is a lower bound on the estimate of the true Na\(^+\),K\(^+\)-ATPase maximal Na\(^+\) extrusion rate since often the pump was working against a continued Na\(^+\) influx, even at the highest intracellular Na\(^+\) concentrations (for example, see the recovery from the second Zero-K\(^+\) inhibition in Fig. 2). There was no obvious relationship between the initial rate of Na\(^+\) extrusion and the
intracellular Na$^+$ concentration just before release of inhibition for [Na$^+$], > 30mM (data not shown).

**Contributions to steady-state Na$^+$ turnover**

We measured the contribution of a specific transport process to steady-state Na$^+$ turnover as the difference between the control, steady-state entry rate (Zero-K$^+$ alone), and the steady-state entry rate measured during application of a specific inhibitor or blocker of the transport process in the same cell (Zero-K$^+$ with inhibitor). Figure 4 shows a schematic diagram of the Na$^+$ entry pathways we investigated.

**Transduction channels.** Hair cells, in vivo, face an apical solution much higher in potassium and lower in sodium ions than in our in vitro environment. Therefore, an obvious source of Na$^+$ entry might be the apical pathway, which contains the mechanosensitive transduction channels. These channels are almost equally permeable to sodium and potassium ions (Corey and Hudspeth, 1979), and are known to have a high conductance (Crawford et al., 1991; Denk et al., 1995; Geleoc et al., 1997), even in the absence of mechanical stimulation (Ricci and Fettiplace, 1998).

Although we exposed the inner ear tissues to EGTA (1mM) during cell dissociation, a treatment which breaks tips links and closes transduction channels (Assad et al., 1991; Crawford et al., 1991), there could still have been some intact channels in our isolated cells. Exposure of the bird auditory papilla to 2mM EGTA for 15 minutes (which is closest to our treatment conditions) left about 10% of tip links intact (Duncan et
al., 1998). We therefore used three independent methods to investigate whether there were still intact transduction channels contributing to Na\(^+\) entry.

To obtain cells with a minimal number of open transduction channels, we took cells prepared by our standard method and examined Na\(^+\) entry rate either: 1) in cells treated with 5mM EGTA for 5 minutes, 2) in the presence of 1mM dihydrostreptomycin, a commonly used blocker of transduction channels, or 3) in cells with the distinctive size and shape of hair cells (Sugihara and Furukawa, 1989), but missing hair bundles.

A one-way analysis of variance showed significant differences between the steady-state Na\(^+\) entry rate in control cells (standard dissociation in 1 mM EGTA) versus all three groups of cells expected to have minimal open transduction channels. In addition, there was no difference in steady-state Na\(^+\) entry rates among any of the three methods used to close or block transduction channels.

In the cells that received the additional EGTA treatment the steady-state Na\(^+\) entry rate was 6.3 ± 0.4 mM/min (n = 29). In cells exposed to 1 mM DHS, the steady-state Na\(^+\) entry rate was 4.8 ± 1.0 mM/min (n = 6). Cells missing hair bundles had a steady-state Na\(^+\) entry rate of 4.8 ± 1.2 mM/min (n=5).

Figure 6 summarizes the Na\(^+\) entry rates in control cells versus all three methods to remove or inhibit transduction channels. By difference, the Na\(^+\) entry via transduction channels in cells isolated with the standard procedure was between 2.6 mM/min (i.e., the difference between the control cells and additional EGTA cells) and 4.1 mM/min (i.e. the difference between control cells and cells missing hair bundles) and represents about 1/3-1/2 of the total control steady-state Na\(^+\) entry rate (9.0 mM/min).
We also monitored \([\text{Na}^+]_i\) while hair cells were exposed to the 5mM EGTA solution for 5 minutes and during 5-10 minutes of recovery. Figure 5 shows a typical experiment in which the Na\(^+\) entry rate (using Zero-K\(^+\) inhibition) was measured both before and after the EGTA treatment. During perfusion of EGTA, \([\text{Na}^+]_i\) increased very rapidly in all cells, even though the pump was not exogenously inhibited during this time. For some cells, the EGTA perfusion included Mg\(^{2+}\) ions, and in these cases \([\text{Na}^+]_i\) also increased, although not as rapidly. In all cases, the \([\text{Na}^+]_i\) recovered fully after washing out the EGTA solution.

**Na\(^+\)/H\(^+\) Exchanger.** The Na\(^+\)/H\(^+\) exchanger, an important homeostatic mechanism for alleviating intracellular acid loads, uses the downhill entry of Na\(^+\) to remove acid equivalents. Although most cell types do display Na\(^+\)/H\(^+\) exchange activity in response to intracellular acid loads, some also have steady-state activity of the Na\(^+\)/H\(^+\) exchanger (Vigne et al., 1984), while others do not (Grinstein and Rothstein, 1986).

To measure steady-state Na\(^+\) entry rate via the Na\(^+\)/H\(^+\) exchanger, we used the fluorescent probe BCECF to measure changes in intracellular pH, after inhibition of the exchanger with ethylisopropyl amiloride (EIPA, 10 or 50 \(\mu\)M). It was not possible to measure \([\text{Na}^+]_i\) during application of EIPA due to large increases in the ultraviolet autofluorescence inside the cell (Negulescu et al., 1990; Hug and Bridges, 2001), which interfered with the SBFI signal. We measured the initial change in pH after blocking the exchanger with EIPA (d\(pH/dt\)) and multiplied this slope by the intrinsic pH buffering capacity of the cell in mM/pH. At a stoichiometry of 1:1, the initial rate of loss of acid
equivalents determined in this way reflects the contribution of Na\(^+\) entry \((d[Na^+]_i/dt)\) via the Na\(^+\)/H\(^+\) exchanger to total steady-state Na\(^+\) entry.

We first measured the steady-state pH\(_i\) and buffering capacity. In 33 cells, pH\(_i\) was 7.38 ± 0.04, which is similar to the steady-state pH\(_i\) in guinea-pig outer hair cells. We measured the intrinsic pH\(_i\) buffering capacity using either ammonium (NH\(_4^+\)) addition, which produces an alkalinization, or ammonium withdrawal, which produces an acidification (Boron and De Weer, 1976). In the example shown in Figure 7, first the pH\(_i\) buffering capacity was measured during ammonium withdrawal and was found to be 37 mM/pH. After recovery from the acidification, the Na\(^+\)/H\(^+\) exchanger was inhibited with EIPA and the initial rate of decrease in pH was 0.13 pH/min, or 4.8 acid equivalents/min after applying the buffering capacity.

The average pH buffering capacity was 21.0 ± 2.4 mM/pH\(_i\) for ammonium addition, and 22.4 ± 2.2 mM/pH\(_i\) for ammonium withdrawal (n=25, same cells for both methods). There was a linear relationship between the pH buffering capacity measured with the ammonium withdrawal method and the pH\(_i\) just before withdrawal, as shown in Figure 8 (slope = -52 mM/pH/pH). The average initial rate of decrease in pH\(_i\) \((dpH_i/dt)\) after application of EIPA (10 or 50 \(\mu\)M) was .065 ± .009 pH/min (n=19). Applying the buffering capacity, estimated from the linear regression curve (Figure 8) and the pH\(_i\) just before application of the EIPA, the steady-state Na\(^+\) entry rate via the Na\(^+\)/H\(^+\) exchanger was 1.99 ± .44 mM/min, or 1/5 of the total steady-state Na\(^+\) entry (9.0 mM/min).

Since for many cell types the exchanger is not active in the steady state, we decided to investigate whether intact transduction channels were contributing an acid permeability. We therefore performed the same type of measurements in cells after the
additional 5mM EGTA treatment and found that the Na\(^+\)/H\(^+\) exchanger rate was not significantly different from control cells (n=6). Hence the transduction channels do not appear to contribute a substantial acid permeability.

**Na\(^+\)/Ca\(^{2+}\) Exchanger.** There is physiological evidence for a Na\(^+\)/Ca\(^{2+}\) exchanger in both guinea-pig outer hair cells (Ikeda et al., 1992a) and type 1 vestibular hair cells (Chabbert et al., 1995). However the steady-state intracellular Na\(^+\) concentration was quite high in both studies (> 85 mM), so that the exchanger was not working in the normal forward direction of Na\(^+\) influx and Ca\(^{2+}\) efflux. Therefore we decided to look for steady-state activity of the exchanger in our hair cells, which have a physiological low [Na\(^+\)]. We used two different approaches to measure the contribution of the exchanger. We measured the effect of directly blocking the Na\(^+\)/Ca\(^{2+}\) exchanger on Na\(^+\) entry rate. Assuming that the exchanger counters Ca\(^{2+}\) influx across the plasma membrane, we also measure the effect of blocking voltage-gated calcium channels on Na\(^+\) entry rate.

We measured the difference in steady-state Na\(^+\) entry rate using Ni\(^{2+}\) as a blocker of the Na\(^+\)/Ca\(^{2+}\) exchanger (Despa et al., 2002; Kopper and Adorante, 2002). As shown in figure 9A, the steady-state Na\(^+\) entry rate decreased significantly in the presence of 5 mM Ni\(^{2+}\). In paired experiments, we measured a decrease in the steady-state Na\(^+\) entry rate of 2.2 ± 0.8 mM/min (n=12) in the presence of 5mM Ni\(^{2+}\) (control rate of 9.1 ± 0.9 mM/min versus 6.9 ± 0.8 mM/min). We also repeated the same type of experiments in cells which had received an additional EGTA treatment to close transduction channels. In 5 cells, the steady-state Na\(^+\) entry rate also decreased in the presence of 5 mM Ni\(^{2+}\).
Hair cells contain L-type, dihydropyridine-sensitive calcium channels blocked by nifedipine (Roberts et al., 1990; Zidanic and Fuchs, 1995). These channels may be involved in the steady-state influx of calcium required for spontaneous discharge since they can sustain large, non-inactivating currents (Martini et al., 2000) and have a fairly negative activation range (Zidanic and Fuchs, 1995). If there is an appreciable steady-state calcium influx across the plasma membrane involved in spontaneous neurotransmitter release, then there may also be an appreciable steady-state Na⁺ entry via the Na⁺/Ca²⁺ exchanger in order to relieve this calcium influx. Therefore, blocking the influx of calcium via nifedipine-sensitive calcium channels should also block Na⁺ entry via the Na⁺/Ca²⁺ exchanger.

Since nifedipine has also been shown to block hair cell mechanotransduction channels (Jorgensen and Kroese, 1995), we only measured the effect of nifedipine in cells after a 5mM EGTA treatment to close transduction channels. Figure 9B shows an example of a cell in which both the steady-state [Na⁺], and Na⁺ entry rate decreased in the presence of nifedipine. On average, the steady-state Na⁺ entry rate decreased by 1.5 ± 0.6 mM/min (n=9) in the presence of 100 μM nifedipine (6.1 ± 0.9 mM/min control versus 4.6 ± 0.9 mM/min nifedipine), a value indistinguishable from the Ni²⁺-sensitive component of Na⁺ entry rate. Therefore the Ni²⁺-sensitive Na⁺ entry most likely represents a Na⁺/Ca²⁺ exchanger which balances Ca²⁺ entry via L-type, voltage-gated, calcium channels.

**Na⁺,K⁺,Cl⁻ cotransport.** There is physiological evidence for Na⁺-K⁺-Cl⁻ cotransport in guinea-pig type I vestibular hair cells (Rennie et al., 1997), assayed by
K⁺-depolarization-induced shape changes. As presented earlier, we found that the Na⁺ entry rate measured with ouabain as a blocker of the pump was 1mM/min faster than the rate measured with Zero-K⁺. One possible reason for the lower entry rate with Zero-K⁺ may be inhibition of the Na⁺,K⁺,2Cl⁻ cotransporter.

To examine whether there is a component of steady-state Na⁺ entry due to this cotransporter, we used 100 μM bumetanide as a blocker. We compared the initial Na⁺ entry rate in cells treated with 150 μM ouabain + 100 μM bumetanide to that in cells treated with ouabain alone (unpaired because ouabain is poorly reversible). There was no difference in Na⁺ entry rates between control (ouabain alone: 9.8 ± 0.8mM/min, n=10) and ouabain with bumetanide (11.4 ± 2 mM/min, n=4), indicating no substantial contribution of the Na⁺,K⁺,2Cl⁻ cotransporter to steady-state Na⁺ entry.

**Na⁺, Amino Acid co-transport.** Hair cells must be able to release neurotransmitter at high rates both at rest and in response to acoustic stimulation. Na⁺-coupled uptake of substrates needed for synthesis of neurotransmitter is a potentially significant basolateral entry pathway.

Ryan and Schwartz (1984) measured uptake of amino acids in the cochlea and found that glutamine was preferentially taken up by inner hair cells. This preferential labeling may be due to the use of glutamine as a substrate for the neurotransmitter at the hair cell afferent synapse. Glutamine can also be an important substrate for energy metabolism in tissues which are poorly vascularized, like the Organ of Corti (i.e. the sensory epithelium of the cochlea). Glutamine transport is often coupled to Na⁺ entry.
(Broer and Brookes, 2001). Na⁺-coupled uptake of other amino acids may also occur in hair cells as it does in other cell types (Cohen and Lechene, 1990).

To measure a Na⁺ entry pathway via Na⁺-coupled amino acid co-transport, we measured the Na⁺ entry rate with and without extracellular amino acids. In 4 cells, the paired decrease in Na⁺ entry rate without amino acids was an insignificant 0.3 ± 0.2 mM/min (6.5 ± 0.7 mM/min control versus 6.2 ± 0.8 mM/min without amino acids). Thus, at least in the steady-state, we did not find significant Na⁺-coupled amino acid transport.

TTX-sensitive channels. Besides the mechanosensitive transduction channels, hair cells have other ion channels that may be a significant source of steady-state Na⁺ entry. In particular tall goldfish hair cells have TTX-sensitive voltage-gated Na⁺ channels (Sugihara and Furukawa, 1989), and in our cell isolation procedure, only these tall hair cells survive (Mroz et al., 1993b). We measured steady-state Na⁺ entry rate with and without 10 μM TTX. In seven cells, there was no paired difference (-0.4 ± 0.5 mM/min) in steady-state Na⁺ entry rate between control cells (7.2 ± 0.9 mM/min) and cells with 10 μM TTX (7.7 ± 0.9 mM/min).

Effect of K⁺ depolarization. Although TTX-sensitive channels did not significantly contribute to steady-state Na⁺ entry, we investigated whether membrane depolarization might increase Na⁺ entry via these channels. In neurons, K⁺ depolarization increased [Na⁺], due to activation of TTX-sensitive Na⁺ channels (Rose and Ransom, 1997a). We decided to investigate the effect of increasing [K⁺], by 20 mM, which depolarized
isolated mammalian outer hair cells by ~40 mV (Sunose et al., 1992). Surprisingly, increasing \([K^+]_o\) decreased the steady-state intracellular Na\(^+\) concentration by 10.6 ± 2 mM (from a control value of 18.4 ± 1.7 mM to 7.7 ± 2.4 mM (n=3)).

To figure out whether the K\(^+\) depolarization-induced decrease in [Na\(^+\)]\(_i\) was due to a decrease in Na\(^+\) entry rate, we measured Na\(^+\) entry rate in the presence of 23.5 mM K\(^+\) using ouabain as an inhibitor of the Na\(^+,K^+\)-ATPase. We measured Na\(^+\) entry rates in control (150 µM ouabain) versus K\(^+\) depolarization (150 µM ouabain + 23.5 mM K\(^+\)) in cells after an additional 5mM EGTA treatment, in order to rule out an effect of intact transduction channels. In two cells the Na\(^+\) entry rate decreased in the presence of the high K\(^+\). Thus the K\(^+\) depolarization-induced decrease in [Na\(^+\)]\(_i\) was most likely due to a reduction in Na\(^+\) entry rate.

The reduction in Na\(^+\) entry could have been due to a decrease in the driving force for Na\(^+\) entry via another, non-TTX channel, or an electrogenic transporter which transports next positive charge into the cell. Another possibility consistent with our results is a reduction of the K\(^+\) gradient across the plasma membrane driving the Na\(^+\)/Ca\(^{2+}\)-K\(^+\) exchanger.

**Hemi-channels and carbenoxolone.** Since goldfish hair cells form gap junctions with supporting cells in vivo (Hama, 1980), we used carbenoxolone to block possible exposed hemi-channels (Kamermans et al., 2001) in the isolated condition. Surprisingly carbenoxolone greatly increased the Na\(^+\) entry rate. The entry rate doubled for 10 and 50 µM of the drug (1 cell for each concentration, example shown in Figure 10) and quadrupled for 100 µM (n=1). In addition carbenoxolone, even at the lowest
concentration (10 μM), increased intracellular Na<sup>+</sup> concentration when perfused in the Standard solution before inhibiting the Na<sup>+</sup>,K<sup>+</sup>-ATPase as shown in Figure 10. This was an unexpected result; we are aware of no reports of this kind of effect of carbenoxolone on [Na<sup>+</sup>].

**DISCUSSION**

Many of the individual currents and conductances in various types of hair cells have been exhaustively characterized by electrophysiological studies, including apical transduction channels and basolateral K<sup>+</sup>- and Ca<sup>2+</sup>- channels. These processes operate on the time scale of milliseconds to shape the response of hair cells to auditory stimulation. Yet the timing and sensitivity of these responses directly depend on the ion homeostatic mechanisms, operating on the time scale of minutes to hours, which maintain the steady-state intracellular ionic environment. Electrophysiological studies are mainly used to study large transient currents (hundreds of pA) in response to large voltage changes (tens of mV), whereas many mechanisms of ion homeostasis involve steady currents with magnitudes of a few tens of pA or less. These “leak” currents of electrophysiology, however, can have enormous consequences for ion homeostasis. A steady inward Na<sup>+</sup> current of just a few tens of pA (equivalent to our maximal Na<sup>+</sup>-pump extrusion rate of 17 mM/min) would over-run the short-term maximal capacity of the Na<sup>+</sup>,K<sup>+</sup>-ATPase.

In addition there are many important homeostatic processes that do not produce a current, such as the Na<sup>+</sup>/H<sup>+</sup> exchanger. In this study, we were able to measure
physiologically relevant processes involved in sodium ion homeostasis in hair cells with an intracellular Na\(^+\) concentration similar to that expected in vivo. We discuss these general issues first, and then the significance of the specific Na\(^+\) entry pathways we have uncovered.

_Goldfish hair cells in vitro maintain a steady-state Na\(^+\) concentration similar to the in vivo value._

The ability to isolate hair cells with low sodium, high potassium, and low free calcium ion concentrations is crucial for studies on ion transporter rates. We used the protocol developed by Mroz et al. (1993b) for isolating hair cells from the inner ear organs of goldfish. This preparation was shown to yield large numbers of hair cells with low intracellular sodium for up to 6 hours after isolation. Use of EGTA was critical during dissociation in order to obtain cells low in sodium.

Table 2 lists steady-state [Na\(^+\)], measured in various types of isolated hair cells. Investigators who have isolated hair cells either from the mammalian cochlea or vestibular system have in general not been able to obtain cells with low steady-state intracellular Na\(^+\) concentrations. Only guinea-pig outer hair cells (OHCs), isolated and maintained in solutions low in sodium (14 mM), had low steady-state levels of intracellular Na\(^+\) concentration (Sunose et al., 1993).

The steady-state [Na\(^+\)], in goldfish hair cells from this study was only slightly lower than measurements made with electron-probe analysis (Table 1), and slightly higher than in vivo measurements in mammalian hair cells (Ikeda and Morizono, 1990). The low steady-state [Na\(^+\)], in isolated goldfish hair cells was also comparable to that found in many other cell types, such as neurons (Rose and Ransom, 1997a) and cardiac
myocytes (Despa et al., 2002). Fluorescence ratio ion imaging in SBFI-loaded goldfish hair cells thus gives reliable values for [Na\(^+\)].

*The steady-state rate of Na\(^+\) entry is fast compared to other cell types.*

The steady-state Na\(^+\) entry rate in goldfish hair cells was remarkably high for an epithelial cell not thought to be involved in active transepithelial ion transport. Figure 12 compares goldfish hair cells to other cell types in this regard. Goldfish hair cell Na\(^+\) entry rates were even faster than in cells of the kidney cortical collecting duct (3.3 mM/min) from rats that had been fed a low sodium diet to increase sodium permeability. In addition, steady-state Na\(^+\) entry rate in fish hair cells was over an order of magnitude faster than in fish hepatocytes (0.2 mM/min, measured using \(^{22}\)Na tracer flux). Except for trout hepatocytes and goldfish hair cells, all others (in Fig 12) were mammalian cells maintained at 37°C. The leakiness of cell membranes in endotherms is usually 3-4 fold that in ectotherms, due to differences in the state of unsaturation of fatty acyl chains (Wu et al., 2001). Thus the high steady-state Na\(^+\) entry rate in goldfish hair cells is even more surprising.

The steady-state Na\(^+\) influx (in pmol/cm\(^2\)/s) can be estimated from the volume to surface ratio of a typical goldfish hair cell (see Methods). The steady-state entry rate of 4.8 mM/min (in cells with missing bundles) would correspond to a Na\(^+\) influx of 13 pmol/cm\(^2\)/s. This estimate of steady-state Na\(^+\) influx is similar to that measured in rat hippocampal neurons of 16 pmol/cm\(^2\)/s (Rose and Ransom, 1997a) and about an order of magnitude larger than in rabbit cardiac myocytes (Despa et al., 2002).
The fast steady-state Na\(^+\) entry measured in isolated goldfish hair cells is physiologically significant.

Two independent methods of measuring steady-state Na\(^+\) entry in goldfish hair cells gave similar fast rates. Our measure of steady-state Na\(^+\) entry (9.0 ± 0.4 mM/min) was higher than that measured with electron-probe analysis (4.4 ± 1.3 mM/min) (Mroz et al., 1993a). However, there are several possible explanations for the lower Na\(^+\) entry rate measured using electron-probe analysis (EPA). The poorer time resolution of EPA-based studies may have missed early changes in sodium concentration. The integrity of individual hair cells could not be determined, therefore the averaging of Na\(^+\) concentration across cells at each time point in the EPA study may have included some cells with compromised Na\(^+\)-pump function (the distribution of [Na\(^+\)], was skewed toward high Na\(^+\)). In addition, the cells as prepared for EPA may have had fewer intact transduction channels than those in the present study.

Any hyperpolarization produced by the Zero-K\(^+\) solution does not lead to an overestimation of the steady-state Na\(^+\) entry rate, since the entry rate based on ouabain inhibition, which should have depolarized the membrane potential, was even higher.

The high rate of Na\(^+\) entry in isolated goldfish hair cells does not appear to be an artifact of exposure of the apical end of the hair cell to a higher Na\(^+\) concentration in vitro than the cells would normally face in vivo. Even in cells missing hair bundles, the Na\(^+\) entry rate was high. It is also unlikely that a new entry pathway has been introduced by the isolation procedure because the cells have a normal, low steady-state intracellular Na\(^+\) concentration (< 13mM). For most cell types, the steady-state Na\(^+\) entry rate is typically at least a third of the maximal Na\(^+\) extrusion rate of the pump (Lechene, 1988).
goldfish hair cells the steady-state entry rate was half of the maximal pump rate, which argues against a substantial artifactual pathway. If there were a major artifactually increased permeability, the Na⁺,K⁺-pump would most likely not have been able to keep up until the cell could synthesize new pumps, a process which would take much longer than the time course of our experiments.

In vivo, there are gap junctions between goldfish hair cells and supporting cells (Hama, 1980). In the brain, gap junctions between astrocytes are thought to contribute to Na⁺ ion homeostasis by equalizing ion concentrations between cells (Rose and Ransom, 1997b). However, goldfish hair cells apparently do not rely solely on their gap junctions with supporting cells to maintain Na⁺ homeostasis, since these hair cells themselves have high Na⁺,K⁺-ATPase activity.

*The steady-state Na⁺ entry rate measured in goldfish hair cells is consistent with “leak” currents in whole-cell patch clamp studies.*

If all of the steady-state Na⁺ entry into goldfish hair cells of 9.0 mM/min were due to a conductance, the Na⁺ current would only be 18 pA. This current is well within the range of the steady-state “leak” current measured in whole-cell patch clamp. For example, Ricci and Fettiplace (Ricci and Fettiplace, 1998) measured a maximum leakage current of 50 pA in hair cells of the turtle basilar papilla. The steady-state rate of the Na⁺-pump in goldfish hair cells is 1/3 of the Na⁺ entry rate (3 Na⁺ ions exported per ATP split) or 3 mM/min. This steady-state Na⁺-pump rate brings in K⁺ ions at a rate of 6 mM/min (2 K⁺ ions imported per ATP split), which would correspond to a K⁺-current of 12 pA. Thus the pump must counter a steady-state K⁺ outward leak current of 12 pA.
Indeed, Sugihara and Furukawa (1996) measured a 10 pA outward K⁺ leak current via inwardly rectifying K⁺-channels in isolated goldfish hair cells.

*Transduction channels represent a significant component of steady-state Na⁺ entry in vitro.*

During the initial step of our cell dissociation procedure, the inner ear tissues were incubated in an EGTA solution (1mM EGTA, 0 Ca²⁺, 0 Mg²⁺) for 20 minutes. We know from previous studies that EGTA or BAPTA (2-5 mM) treatment of tissues (Zhao et al., 1996; Duncan et al., 1998) or isolated hair cells (Assad et al., 1991; Crawford et al., 1991; Meyer et al., 1998) disrupts tip links and abolishes mechanotransduction currents. To investigate whether there were still some intact transduction channels in our isolated cells contributing to steady-state Na⁺ entry we employed three independent treatments to eliminate or block transduction channels (additional EGTA treatment, 1mM dihydrostreptomycin, missing bundles). All three methods indicate Na⁺ entry rates of about 3mM/min via the transduction channels that had not been closed by the standard EGTA treatment. Hence the remaining transduction channels represent about 1/3 of the total steady-state Na⁺ entry rate in goldfish hair cells isolated by our standard treatment.

One study suggests that although BAPTA treatment eliminates mechanically-gated currents in hair cells, a steady-state inward current develops due to permanently opened transduction channels (Meyer et al., 1998). Crawford et al. (1991) showed that although whole cell inward current increases soon after treatment with low Ca²⁺ (< 1μM), the response is only transient and then the current subsequently returns to control levels. The inward current in the absence of extracellular Ca²⁺ may also include Na⁺ entry via
calcium channels (discussed later). In addition, another technique for quantifying the steady-state conductance via transduction channels, rapid apical uptake of the dye FM-143 (Gale et al., 2001), showed that after treatment with 5 mM EGTA uptake of the FM-143 was reduced, which is consistent with EGTA closing rather than opening transduction channels. In goldfish hair cells, the entry rate in cells which received the additional EGTA treatment was not different from the rates measured in cells with missing bundles. Thus it appears that the additional 5 mM EGTA treatment did eliminate essentially all remaining transduction channels.

*The measured Na\textsuperscript{+} entry via transduction channels is consistent with estimates of steady-state conductance and may be similar to the in vivo rate*

Exposure of the bird auditory papilla to 2 mM EGTA for 15 minutes (which is closest to our treatment conditions during cell dissociation) left about 10% of tip links intact (Duncan et al., 1998). The 3 mM/min Na\textsuperscript{+} entry rate we measured, which would correspond to a 6 pA current, is entirely consistent with 10% of the transduction channels being intact in our isolated cells. The single-channel open conductance of a hair cell is estimated to be about 100 pS (with a reversal potential of 0 mV) and there are about 100 channels per bundle (of which we assume only 10% are intact in our preparation). If we also assume that 10% of these intact channels are open at rest (i.e., only a single open transduction channel), and that the steady-state membrane potential ($V_m$) is –80 mV (Sugihara and Furukawa, 1989), then steady-state current via transduction channels would be 8 pA, which is close to the 6 pA (3 mM/min) we predicted from Na\textsuperscript{+} entry via
transduction channels. Remarkably, even a single open transduction channel can
contribute a significant portion to the total steady-state Na\(^+\) entry.

The Na\(^+\) entry via transduction channels in our in vitro preparation (~10% intact
transduction channels in a high Na\(^+\) medium) may be similar to that faced by fish hair
cells in vivo (100% intact transduction channels and a low Na\(^+\) concentration in
endolymph). The 6 pA of transduction channel Na\(^+\)-current we predicted in our isolated
cells corresponds to a conductance of 43 pS. In vivo, with all transduction channels
intact, the conductance would be 430 pS. The Na\(^-\) current via these channels would be
scaled by the relative concentrations of Na\(^+\) to K\(^+\) in fish endolymph. Using a lower-
bound estimate of endolymph Na\(^+\), based on the concentrations in the mammalian semi-
circular canal, the ratio of Na\(^+\)/K\(^+\) concentrations is about 0.1 (15 mM Na\(^+\), 144 mM K\(^+\)).
If we assume that the endolymphatic potential in vivo is 10mV, \(V_m = -80\) mV, and \(V_{Na} =
0mV\), then the transduction current carried by Na\(^+\) in vivo would be 4 pA. This value is
close to our estimate of 6 pA in the cells in vitro, with high apical Na\(^+\) and 90% damaged
transduction channels.

Thus the steady-state Na\(^+\) entry rate via transduction channels in our in vitro
preparation is similar to the steady-state rate in vivo. In addition, a lower bound on the
total basolateral steady-state Na\(^+\) entry rate of 4.8 mM/min (in hair cells missing hair
bundles) probably also represents the in vivo total basolateral Na\(^+\) entry.

In isolated hair cell preparations in a high [Na\(^+\)]\(_o\) medium, Na\(^+\) influx via
transduction channels may overwhelm Na\(^+\) homeostasis if the cells are not explicitly
treated to close transduction channels.
Goldfish hair cells, unlike guinea-pig outer hair cells, have steady-state activity of the Na$^+$/H$^+$ exchanger.

In many cell types the Na$^+$/H$^+$ exchanger is quiescent at steady-state pH$_i$, and is activated by acidification (Aronson, 1985; Frelin et al., 1986; Grinstein and Rothstein, 1986). In such cells, the Na$^+$/H$^+$ exchanger can be stimulated by mitogens, tumor promoters, hormones, or hyperosmotic shock (Grinstein and Rothstein, 1986). The stimulation shifts the pH$_i$ dependence of the exchanger in the alkaline direction.

In goldfish hair cells, however, we found that about 1/5 of the total steady-state Na$^+$ entry was due to an EIPA-sensitive Na$^+$/H$^+$ exchanger operating at a steady-state pH$_i$ of 7.38. This differs from isolated guinea-pig outer hair cells (Ikeda et al., 1992b), where neither addition of amiloride nor replacement of extracellular Na$^+$ with NMDG (N-methyl-D-glucamine) affected steady-state pH$_i$, indicating that the Na$^+$/H$^+$ exchanger was not active at rest in these cells. The exchanger in guinea-pig outer hair cells was activated by intracellular acidification.

Other cell types that also have steady-state activity of the Na$^+$/H$^+$ exchanger include cardiac myocytes (Despa et al., 2002), skeletal muscle (Vigne et al., 1984; Juel, 1998), kidney mesangial cells (Boyarsky et al., 1990), hippocampal neurons (Raley-Susman et al., 1991), gastric cells (Negulescu et al., 1990), and trout hepatocytes (Furimsky et al., 1999). In rat cardiac myocytes, the Na$^+$/H$^+$ exchanger accounted for about 1/4 of total steady-state Na$^+$ entry. In trout hepatocytes, an even larger fraction of Na$^+$ entry was attributed to the exchanger (1/2), but the absolute magnitude of entry was an order of magnitude slower than what we measured in goldfish hair cells.
The intrinsic buffering capacity we measured was virtually the same whether we used the ammonium addition or withdrawal technique. Our estimate of average pH buffering capacity at steady-state pH$_i$ (~21 mM/pH) was not much different from that measured in guinea-pig outer hair cells (15 mM/pH).

*Steady-state Na$^+/H^+$ activity may be a hallmark of highly active cells*

The steady-state Na$^+/H^+$ activity may either be used to extrude intracellularly derived protons, produced by metabolism, or to counter passive proton entry across the plasma membrane. The electro-chemical gradient across most cell membranes favors proton entry (Roos and Boron, 1981). In particular, the intracellular pH of goldfish hair cells is 0.15 pH units more acidic than the extracellular fluid, but is more alkaline than would be expected for a passive distribution of protons across the plasma membrane. In addition, goldfish hair cells have a high proton permeability, such that extracellular changes in pH$_o$ cause very rapid changes in pH$_i$ (Kusakari, Lechene, Mroz, personal communication). This is very different from the situation in guinea-pig outer hair cells, which were relatively insensitive to extracellular pH changes (Ikeda et al., 1992b). The high proton permeability can account for a passive entry of protons across the plasma membrane at rest, which matches the extrusion rate of the Na$^+/H^+$ exchanger, without having to include a component of proton production from metabolism. Trout hepatocytes are similar to goldfish hair cells in having a proton permeability countered by steady-state activity of the Na$^+/H^+$ exchanger, although the rates are an order of magnitude smaller in the trout.
The benefit of expending cell energy to counter a steady-state acid permeability is unclear. However, all of the cell types mentioned above which exhibit steady-state Na\(^+\)/H\(^+\) activity might be exposed to large perturbations in intracellular pH, either due to intense activity (skeletal and cardiac muscle, or neurons) or due to the external pH environment (gastric cells). Goldfish hair cells (and trout hepatocytes) may also be exposed to large fluctuations in acid/base status in vivo due to changes in O\(_2\) availability. Steady-state Na\(^+\)/H\(^+\) exchanger activity may either minimize the intracellular pH changes or allow these cells to respond more quickly to such disturbances.

*The Ni\(^{2+}\)-sensitive component is most likely the Na\(^+\)/Ca\(^{2+}\) Exchanger*

Evidence for Na\(^+\)/Ca\(^{2+}\) exchange has been shown in guinea-pig type I vestibular hair cells (Chabbert et al., 1995) and outer hair cells (Ikeda et al., 1992a). In these studies, removal of external Na\(^+\) by replacement with NMDG caused a reversible rise in intracellular calcium, presumably due to "backwards" operation of the exchanger. The increase in intracellular Ca\(^{2+}\) was inhibited 63% by 100 \(\mu\)M amiloride 5-(N,N-dimethyl) hydrochloride (DMA or dimethyl amiloride), an inhibitor of Na\(^+\)-coupled transporters with a higher affinity for the Na\(^+\)/Ca\(^{2+}\) exchanger. The exchanger did not appear to be active in those studies at rest, as treatment with 100 \(\mu\)M DMA on its own did not change intracellular calcium concentration. Indeed since the steady-state intracellular Na\(^+\) concentration was so high in those studies, the exchanger could not have been working in the forward direction of Na\(^+\) influx and Ca\(^{2+}\) efflux. In vestibular type 1 hair cells, there is both physiological and immunohistochemical evidence that the exchanger is a Na\(^+\)/Ca\(^{2+}\)-K\(^+\) exchanger (Boyer et al., 1999), like that in rod photoreceptors (Cervetto et
al., 1989; Schnetkamp et al., 1989), with a stoichiometry of 4 Na\(^+\) exchanged for 1 Ca\(^{2+}\) and 1 K\(^+\).

Other investigators have used Ni\(^{2+}\) as a probe for steady-state Na\(^+\) entry via the Na\(^+\)/Ca\(^{2+}\) exchanger. In rat cardiac myocytes (Despa et al., 2002) a Ni\(^{2+}\)-sensitive component accounted for about 37% of total steady-state Na\(^+\) entry. Although 5 mM Ni\(^{2+}\) is not necessarily a selective inhibitor of the exchanger (Blaustein and Lederer, 1999), the magnitude of calcium efflux via the Na\(^+\)/Ca\(^{2+}\) exchanger (i.e. the Ni\(^{2+}\)-sensitive component) measured by Despa and colleagues agreed with independent measurements of steady-state Ca\(^{2+}\) efflux and influx in the same cell type (Choi et al., 2000).

We found that Ni\(^{2+}\) decreased the steady-state Na\(^+\) entry rate by about 2 mM/min. If this effect were due to blocking Na\(^+\) entry via the Na\(^+\)/Ca\(^{2+}\)-(K\(^+\)) exchanger, at an Na\(^+\):Ca\(^{2+}\) stoichiometry of 4:1, then the steady-state Ca\(^{2+}\) efflux via the exchanger would be 0.5 mM/min. A steady-state influx of Ca\(^{2+}\) of 0.5 mM/min via voltage-gated calcium channels, countered by Na\(^+\)/Ca\(^{2+}\)-(K\(^+\)) exchange would produce a Ca\(^{2+}\) current of 2 pA. This is a reasonable value for Ca\(^{2+}\) current near the steady-state \(V_m\). Roberts et al. (1990) estimated that the voltage-gated calcium current is activated at a level of 2% at rest; this would require a maximum voltage-gated calcium current of 100 pA, which is well within the range of such currents measured in various hair-cell types (Zidanic and Fuchs, 1995; Martini et al., 2000).

Another piece of evidence supports the idea that the Ni\(^{2+}\)-sensitive component represents steady-state activity of a Na\(^+\)/Ca\(^{2+}\)-(K\(^+\)) exchanger, which is used to counter Ca\(^{2+}\) influx via voltage-gated calcium channels. Nifedipine is a blocker of L-type calcium channels. Hair cells possess L-type calcium channels, which are unusual in
having a fairly negative activation range (Hudspeth and Lewis, 1988; Zidanic and Fuchs, 1995). The channels may contribute to the influx of calcium, which triggers spontaneous as well as stimulus-evoked neurotransmitter release. We found a nifedipine-sensitive component of steady-state Na\(^+\) entry rate of 1.5 mM/min, which was indistinguishable from the Ni\(^{2+}\)-sensitive component (2 mM/min). The nifedipine-sensitive component may underestimate Ca\(^{2+}\) entry/exit since only L-type calcium channels, not the R-type (Martini et al., 2000), are blocked by the drug. Nifedipine can also block transduction channels, with an IC50 of 1.9 \(\mu\)M (Jorgensen and Kroese, 1995). However we tested the effect of nifedipine in cells which had received an additional EGTA treatment to close transduction channels.

Other candidates for the Ni\(^{2+}\)-sensitive component are unlikely. A Ni\(^{2+}\)-sensitive component of \(~\)2 mM/min was also seen in EGTA-treated cells (n = 5, data not shown), thus we can rule out transduction channels. Voltage-gated Na\(^+\) currents can be blocked by divalent and trivalent cations in the mM range. 5 mM Nickel, however, did not block TTX sensitive Na\(^+\) currents in guinea-pig ventricular myocytes (Levesque et al., 1994), and we found no significant TTX-sensitive component of steady-state Na\(^+\) entry in goldfish hair cells.

Thus we have measured a significant component of steady-state Na\(^+\) entry via a Na\(^+\)/Ca\(^{2+}\)-(K\(^+\)) exchanger, which may be involved in countering a steady-state influx of calcium ions. Since goldfish hair cells are known to release neurotransmitter at rest (Furukawa, 1986; Starr and Sewell, 1991), producing high rates of spontaneous activity in auditory nerve fibers, it is not surprising that we have measured a component of Na\(^+\) entry due to that exchanger.
As opposed to cardiac myocytes, goldfish hair cells do not have a significant component of steady-state Na⁺ entry via TTX-sensitive Na⁺ channels.

Although they are known to exist in goldfish hair cells (Sugihara and Furukawa, 1989), we did not find a significant component of steady-state Na⁺ entry via TTX sensitive channels. The effect of TTX on Na⁺ entry rate was measured using Zero-K⁺, which may have hyperpolarized the membrane potential, causing deactivation of the voltage-gated Na⁺ channels. However, rat cardiac myocytes have a significant TTX-sensitive component of steady-state Na⁺ entry (40%), and such a component was measured in that cell type by inhibiting the Na⁺-pump with Zero-K⁺ (Despa et al., 2002). Hence our methods did not necessarily mask the TTX component. In addition, K⁺ depolarization, which might be expected to activate (at least transiently) TTX channels and increase [Na⁺]ᵢ, as found in hippocampal neurons (Rose and Ransom, 1997a), actually decreased Na⁺ entry in our goldfish hair cells.

*K⁺-depolarization rapidly decreased intracellular [Na⁺]ᵢ.*

Cell types differ in effects of K⁺-depolarization on [Na⁺]ᵢ. Elevation of [K⁺]₀ from 3 to 40 mM in rat hippocampal neurons caused increases in [Na⁺]ᵢ, which could be blocked with TTX (Rose and Ransom, 1997a). In contrast, a [K⁺]₀ of 40 mM did not change intracellular Na⁺ concentration in vascular smooth muscle cells (Borin et al., 1993) and 35 mM K⁺-depolarization of spinal cord astrocytes (Rose et al., 1997) either
caused no change in Na$^+$ concentration (12 of 18 cells), or a slight increase (5 mM, 5 of 18).

Thus our results in goldfish hair cells, a decrease of steady-state [Na$^+$], during depolarization by 20 mM K$^+$, are quite different from the effects reported in other excitable cells. If the Na$^+$/Ca$^{2+}$-(K$^+$) exchanger is a significant component of steady-state Na$^+$ entry, then elevation in [K$^+$]$_o$ may decrease Na$^+$ influx by reducing the outward K$^+$ gradient for the exchanger. Since depolarization of the membrane potential will also reduce the driving force for net cation entry, this component may also represent a non-TTX Na$^+$ channel or a Na$^+$-coupled electrogenic transporter (such as the Na$^+$/Ca$^{2+}$-K$^+$ exchanger).

Thus, based on our findings with elevation of [K$^+$]$_o$ and TTX, voltage-sensitive Na$^+$ channels do not appear to be a significant pathway for steady-state Na$^+$ entry in these hair cells, possibly because these are rapidly inactivating channels.

**Removing divalent cations caused very large increases in [Na$^+$]$_i$.**

As shown in Figure 6, removal of divalent cations caused a large increase in intracellular Na$^+$ concentration, which persisted until washout and then recovered quickly. This result is consistent with whole-cell patch clamp studies on hair cells which measured a persistent inward current during BAPTA or EGTA treatment (Assad et al., 1991; Crawford et al., 1991; Meyer et al., 1998). The increase in Na$^+$ concentration might be due to a massive Na$^+$ influx via L-type calcium channels, which would ordinarily be impermeant to Na$^+$ ions at the normal extracellular Ca$^{2+}$ concentration of 1.2 mM (Hess et al., 1986). In vascular smooth muscle cells Na$^+$ concentration also
increased during removal of divalents, and this increase was prevented by the L-type Ca\(^{2+}\) channel antagonist nitrendipine (Borin et al., 1993).

*Carbenoxolone had unexpected effects on steady-state \([Na^+]_i\) and Na\(^+\) entry*

We used carbenoxolone as a blocker of hemi-channels, since goldfish hair cells in vivo are known to contain gap junctions. However, rather than blocking Na\(^+\) entry, this drug caused large increases in steady-state Na\(^+\) concentration, without exogenous pump inhibition. Carbenoxolone also increased the Na\(^+\) entry rate and hence the effect on [Na\(^+\)]\(_i\) was not due to an inhibition of the Na\(^+\),K\(^-\)-ATPase (Figure 10). One study did report an increase in hippocampal neuron excitability, which is consistent with an increase in Na\(^+\) permeability (Jahromi et al., 2002). However, no studies have reported the effects of this drug on intracellular Na\(^+\) concentration or influx, although it is commonly used in studies of gap junctional communication in the nervous system (Traub et al., 2002).

*We have accounted for \(\frac{3}{4}\) of total steady-state Na\(^+\) entry*

Figure 11 summarizes the quantitative contribution of each significant pathway to total steady-state Na\(^+\) entry. If the EGTA-, EIPA-, and Ni\(^{2+}\)-sensitive pathways are all independent, then \(~3/4\) of the total steady-state Na\(^+\) entry can be accounted for by the combination of transduction channels, Na\(^+\)/H\(^+\) exchanger, and the Na\(^+\)/Ca\(^{2+}\) exchanger. The nifedipine-sensitive component is probably part of the Na\(^+\)/Ca\(^{2+}\) exchanger pathway, since nifedipine blocks Ca\(^{2+}\)-channels. The K\(^+\)-sensitive component (discussed in more
detail below) may either be an ion channel, an electrogenic transporter which transports net positive charge into the cell, or a K⁺-dependent Na⁺/Ca²⁺ exchanger, like the one in type 1 vestibular hair cells (Boyer et al., 1999).

What pathways might account for the remaining ¼ of steady-state Na⁺ entry?

Although we did not find significant Na⁺ entry via TTX channels, Na⁺,K⁺,Cl⁻ cotransport, or Na-Amino Acid transport, it does not mean that these pathways do not contribute to Na⁺ entry. Based on the standard errors of our measurements, we cannot detect a change in Na⁺ entry rate of less than 1mM/min. Thus the sum of these pathways may account for the remaining ¼ of steady-state Na⁺ entry.

Mammalian cochlear hair cells contain apically-located, ATP-gated P2X receptors (Housley et al., 1999). In hair cells surrounded by a high Na⁺ medium, these receptors can cause large changes in intracellular Na⁺ concentration when activated by ATP (Housley et al., 1998). However, we continuously perfuse our isolated cells at a rate of 500 µl/min, so it is unlikely that ATP released from dying cells could be at a high enough local concentration to activate these receptors, if they do exist in goldfish hair cells.

In-situ hybridization studies in the rat inner ear found evidence of epithelial Na⁺ channel (ENaC) in the apical sensory epithelium of the utricle, saccule, and ampullary crest, but it was not possible to resolve whether the expression was in the sensory or supporting cells. Thus there remains the possibility that goldfish hair cells do have apically-located epithelial Na⁺ channels, which may account for some of the remaining
Na⁺ entry. We were not able to look directly for these channels because the inhibitor, amiloride, causes fluorescence interference problems with SBFI, even at the low concentrations required for ENaC inhibition (10 µM).

*Fish hair cells may be better able to deal with Na⁺ loads than are mammalian hair cells.*

Matsuura (1971) found that perfusion of high Na⁺ into the endolymph of the goldfish saccule had little effect on the cochlear microphonic, a measure of hair cell responses to sound, whereas in the guinea pig the cochlear microphonic rapidly decreased when the endolymph was exposed to a high Na⁺ concentration (Konishi et al., 1966). Matsuura and colleagues suggested that the differences in vulnerability to apical Na⁺ between the guinea pig and fish might have to do with fish having almost equal portions of Na⁺ and K⁺ in the endolymph (Enger, 1964). This idea has also been suggested by other studies on fish endolymph composition (Payan et al., 1997; Payan et al., 1999), but remains controversial as it can be difficult to sample the endolymph without causing contamination by perilymph. Even assuming a lower bound on fish endolymph Na⁺ concentration of 15 mM, based on the mammalian vestibular system (Enger, 1964), there would be a substantial apical Na⁺ load via transduction channels in goldfish hair cells in vivo. Hence fish hair cells may be pre-adapted to deal with large apical Na⁺ loads by having higher (basolateral) Na⁺,K⁺-ATPase activity.

We measured a maximal Na⁺ extrusion rate of the Na⁺,K⁺-ATPase of 17 mM/min, which corresponds to an efflux of ~50 pmol/cm²s. Assuming the maximal turnover rate of the Na⁺-pump is 100 per second, then the density of Na⁺,K⁺-ATPase sites would be
1000/µm². Burnham and Stirling (1984a) estimated the density of Na⁺,K⁺-ATPase in bullfrog saccular hair cells to be 3000 sites/µm², based on measures of ouabain binding sites. We may have underestimated the maximal Na⁺ extrusion rate of the Na⁺,K⁺-ATPase since the initial rate of decrease in [Na⁺], after release from pump inhibition also includes a passive influx component. Burnham and Stirling (1984b) also investigated bullfrog saccular dark cells, which are involved in K⁺ recirculation, and the pump density was only 1.5x that in bullfrog saccular hair cells (4500 sites/µm²). In the mammalian ear, on the other hand, immunohistochemical studies suggest that Na⁺,K⁺-ATPase staining in the dark cells or strial marginal cells is orders of magnitude greater than in hair cells (Schulte and Adams, 1989; Spicer et al., 1990).

Thus mammalian outer hair cells may have given up a high Na⁺,K⁺-ATPase capacity in order to emphasize other functions, such as the mechanism of electromotility. Indeed a dense packing of particles in the outer hair cell membrane (Forge, 1991), which most likely represent the motor protein prestin (Zheng et al., 2000), may preclude a high density of Na⁺-pumps. This tradeoff may partly explain why mammalian outer hair cells are so vulnerable to pathophysiological perturbations such as anoxia, acoustic trauma, or ototoxic drugs.
CAPTIONS, TABLES, AND FIGURES

Table 1: Composition of perfusion solutions, in mM. All solutions (except pH cal) also contained, in mM: 20 Hepes, 1 NaH₂PO₄, 0.5 Na₂SO₄, 5 D-Glucose, 4 L-Glutamine (added on day of use). MEM Essential Amino Acids (50x, 1 mL/50 mL), and MEM Non-Essential Amino Acids (100x, 1 mL/100mL) were also added and the final solution adjusted to pH 7.5 with NaOH. The pH cal solution contained 5mM D-glucose, 10mM Hepes, and pH was adjusted with HCl or KOH.

Table 2: Reported steady-state intracellular sodium concentrations in isolated hair cell preps.

Figure 1: Typical calibration for intracellular Na⁺ concentration. A. The time course of the individual F340 and F380 emissions, as well as their ratio (F340/F380) inside a region of interest (ROI) in the cell during a calibration. The pore-forming antibiotic gramicidin, at 5µM, was perfused throughout the calibration to clamp the intracellular Na⁺ concentration to the extracellular concentration. B. The relationship between the average ratio inside the cell and the intracellular Na⁺ concentration (assuming [Na⁺]ᵢ = [Na⁺]ₒ). C. Phase contrast images of two isolated goldfish hair cells. D. Fluorescence emission from the same cells, loaded with SBFI and excited at 380nm. E. Ratio of 340 nm image and 380 nM images (C), after background subtraction. The circles represent the typical size and location of the ROI used to monitor [Na⁺]ᵢ changes. Note the uniformity of the ratio values inside the cells, despite the inhomogeneous fluorescence of the individual wavelengths (C).
Figure 2: Estimating the steady-state Na\(^+\) entry rate and the maximal Na\(^+\)-pump rate. Intracellular Na\(^+\) concentration in 2 cells during two consecutive Na\(^+\)-pump inhibition (using Zero-K\(^+\)) and recovery treatments. During the first Na\(^+\)-pump inhibition, the initial rate of increase in [Na\(^+\)]\(_i\), an estimate of the steady-state Na\(^+\) entry rate, was \(\sim\)12 mM/min for the two cells. The second Na\(^+\)-pump inhibition was longer so that [Na\(^+\)]\(_i\) reached a value \(>\) 80 mM. During recovery from the second Na\(^+\)-pump inhibition in these Na\(^+\)-loaded cells, the initial rate of decrease in [Na\(^+\)]\(_i\) of 19 mM/min provides an estimate of the maximal Na\(^+\) extrusion rate of the Na\(^+\)-pump.

Figure 3: Comparison of two different methods to inhibit the Na\(^+\)-pump: Zero-K\(^+\) or ouabain. A representative Na\(^+,K\(^+\)-pump inhibition and recovery experiment in which the pump was first inhibited with a Zero-K\(^+\) solution, allowed to recover, and then inhibited with 150 \(\mu\)M ouabain, which is poorly reversible. Responses of 4 cells in the same field of view.

Figure 4: Na\(^+\) influx and efflux pathways investigated in this study and their normal steady-state directions of transport, assuming low [Na\(^+\)]\(_i\) (i.e. \(<\) 20 mM) and high [Na\(^+\)]\(_o\) (> 100 mM). The influx pathways include: transduction channels (the only apical pathway), Na\(^+\)/H\(^+\) exchanger (1:1 stoichiometry), Na\(^+\)/Ca\(^2+\) exchanger (3:1 or 4:1 stoichiometry for the Na\(^+\)/Ca\(^2+\)-K\(^+\) exchanger), TTX-sensitive Na\(^+\) channels, Na\(^+\)-Amino Acid cotransport, hemi-channels exposed by dissociation (which would normally be part of gap junctions with supporting cells in vivo), and Na\(^+,K\(^+\),Cl\(^-\) cotransport (1:1:2.
stoichiometry). The Na\textsuperscript{+},K\textsuperscript{+}-ATPase is the only efflux pathway (at the concentrations of Na\textsuperscript{+} mentioned above) and requires energy to actively extrude 3 Na\textsuperscript{+} ions and import 2 K\textsuperscript{+} ions per ATP.

Figure 5: Comparison of Na\textsuperscript{+} entry rates among three different treatments to remove or inhibit transduction channels. The columns are the initial Na entry rate (mM/min) just after pump inhibition in control cells, versus cells which had received an additional treatment to close or inhibit transduction channels. The * indicates a significant decrease from the control entry rate. DHS is dihydrostreptomycin. The Na\textsuperscript{+} entry rates did not differ significantly among the three treatments.

Figure 6: Intracellular Na\textsuperscript{+} concentration in two cells in response to pump inhibition (Zero-K\textsuperscript{+}) before and after a 5mM EGTA treatment (5 minutes, no Mg\textsuperscript{2+} or Ca\textsuperscript{2+}) to close transduction channels. During the perfusion of the EGTA solution, [Na\textsuperscript{+}]\textsubscript{i} increased dramatically, even though the Na\textsuperscript{+}-pump was not exogenously inhibited.

Figure 7: Measurement of pH\textsubscript{i} buffering capacity, using the NH\textsubscript{4}Cl pre-pulse technique (Boron and De Weer, 1976), and steady-state rate of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger from changes in pH\textsubscript{i} after application of EIPA. The intrinsic pH buffering capacity can be calculated from the change in pH\textsubscript{i} either during the NH\textsubscript{4}\textsuperscript{+} addition or withdrawal (see Methods). Based on the acidification during NH\textsubscript{4}\textsuperscript{+} withdrawal (ΔpH = 0.55), the buffering capacity (β\textsubscript{i}) for this cell was 37 mM/pH. After recovery from the acid load, 10 μM EIPA was applied to block the Na\textsuperscript{+}/H\textsuperscript{+} exchanger. The initial rate of pH decrease (0.13 pH/min)
was converted to the increase in mM acid equivalent/min (4.8 mM/min) via the buffering capacity. The 4.8 mM/min increase in acid equivalents/min just after EIPA application represents the steady-state rate at which the Na⁺/H⁺ exchanger must have been extruding acid equivalents just before the inhibition.

Figure 8: The relation between the intrinsic pH buffering capacity measured using the ammonia withdrawal method and the intracellular pH just before withdrawal. A linear fit to the data gave an R² value of 0.72 and a slope of -51.5 mM/pH/pH.

Figure 9: Contribution of the Na⁺/Ca²⁺ exchanger to steady-state Na⁺ entry. Both control Na⁺ entry rates and the rates in the presence of blockers were measured in the same cells. Figure 9A, effect of blocking the Na⁺/Ca²⁺ exchanger on Na⁺ entry rate. The Na⁺ entry rate for this cell decreased significantly in the presence of 5 mM Ni²⁺, a blocker of the exchanger. Figure 9B, effect of blocking L-type calcium channels on steady-state Na⁺ entry rate. Both [Na⁺]i and Na⁺ entry rate decreased in the presence of 100 μM nifedipine, a blocker of L-type calcium channels.

Figure 10: Effect of 10 μM carbenoxolone on steady-state [Na⁺]i and Na⁺-entry rate. Note that [Na⁺]i increased with carbenoxolone alone, before the pump was inhibited.

Figure 11: Significant Na⁺-entry pathways in goldfish hair cells. The entry via transduction channels was measured as the difference between the control entry rate and the rate after cells were treated with 5 mM EGTA for 5 minutes to close the remaining
transduction channels. The rate of entry via the Na⁺/H⁺ exchanger was measured by multiplying the initial rate of pH decrease after application of 10 or 50 μM EIPA with the pH buffering capacity. The Na⁺/Ca²⁺ exchanger rate was measured as the difference between Na⁺ entry rates measured in paired controls versus 5mM Ni²⁺. The nifedipine sensitive component was measured (in 5mM EGTA-treated cells) as the difference between Na⁺ entry rates measured in paired control versus 100 μM nifedipine. The K⁺ sensitive component was measured (in 5mM EGTA-treated cells) as the difference between Na⁺ entry rates measured in controls (150 μM ouabain, 3.5 mM K⁺) versus 20 mM K⁺ depolarization (150 μM ouabain, 23.5 mM K⁺).

Figure 12: Comparison of the steady-state Na⁺ entry rates reported in various cell types. For all cells except the trout hepatocytes, [Na⁺]i was measured with SBFI and the initial rate of Na⁺ entry was measured during Na⁺ pump inhibition (Zero-K⁺ or ouabain). For the rat and trout hepatocytes the Na⁺ entry rates were measured with ²²Na isotope tracer. All experiments were carried out at 37°C for mammalian cells and at room temperature for fish. The Na⁺ entry rates for goldfish hair cells either prepared in the standard way (control, in gray) or exposed to the additional EGTA treatment (post EGTA, in gray) were higher than in kidney cortical collecting duct (CCD) cells (in gray) in rats fed a low Na⁺ diet to increase the Na⁺ permeability of the apical membrane. In addition, the steady-state Na⁺ entry rate in trout hepatocytes (in gray) was over an order of magnitude lower than in goldfish hair cells. The references for each cell types are as follows: Gerbil Strial Marginal cells (Furukawa et al., 1996), Hamster ATL of Henle’s Loop (Kondo et al., 1993), Mouse Cortical Astrocytes (Chatton et al., 2000), Rat Sublingual Mucous
Acini (Zhang et al., 1993), Rat Hippocampal Neurons (Rose and Ransom, 1997a), Rat Submandibular Salivary Duct cells (Xu et al., 1995), Rat Hippocampal Astrocytes (Rose and Ransom, 1996), Rat Hepatocytes (Fitz et al., 1991), Rat Kidney CCD (cortical collecting duct) (Schlatter et al., 1996), Rabbit Parietal Cells (Negulescu et al., 1990), Human Skeletal Muscle (Benders et al., 1994), Rat Ventricular Myocytes (Despa et al., 2002), Mouse Pancreatic β-Cells (Gilon and Henquin, 1993), Rat Embryo Fibroblasts (Harootunian et al., 1989), A7r5 Vascular Smooth Muscle (Borin et al., 1993), Isolated Trout Hepatocytes (Furimsky et al., 2000).
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<th>EGTA- Std</th>
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Table 1
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* in Na$^+$-free medium

Table 2
Figure 1.
Figure 2.
Figure 3.
Figure 5.
Figure 6.
Figure 7.

$\Delta \text{pH} = 0.55$, $\beta_i = 37 \text{ mM/pH}$

d$\text{pH}/dt = 0.13 \text{ pH/min}$

d$/dt$ Acid Equivalents $= 4.8 \text{ mM/min}$
Figure 8.
Figure 10
Figure 11.
Chapter 4: Interactions between \([Na^+]_i\) and pH during an imposed intracellular acid load in isolated goldfish hair cells

ABSTRACT

We examined interactions between regulation of intracellular Na\(^+\) concentration and pH in isolated goldfish hair cells during imposed intracellular acid loads. Fluorescence ratio ion imaging was used to measure intracellular sodium concentration ([\(Na^+\)]) or pH in cells loaded with the indicators SBFI and BCECF, respectively. The rates of Na\(^+\) entry and acid extrusion via the Na\(^+\)/H\(^+\) exchanger were measured during acute intracellular acid loads of 0.6 pH units and were found to be equivalent, after considering the measured pH buffering capacity. The total Na\(^+\) entry rate doubled, and Na\(^+\) entry via the Na\(^+\)/H\(^+\) exchanger increased by a factor of 6, during recovery from the intracellular acidification. In most cells, \([Na^+]_i\) increased immediately after the acid load and subsequently recovered, even though the Na\(^+\),K\(^+\)-ATPase (Na\(^+\)-pump) was not exogenously inhibited. Thus, it appears that the Na\(^+\),K\(^+\)-ATPase may be transiently inhibited at low pH\(_i\). The actual changes in intracellular \([Na^+]_i\) depend on the quantitative balance of two opposing influences: the tendency of acidification to inhibit the pump, and the tendency of increased \([Na^+]_i\) to activate the pump. The temporary loss of Na\(^+\) ion homeostasis during an intracellular acid load may be a crucial component of injury to hair cells during such pathophysiological insults as anoxia or ischemia.
INTRODUCTION

Maintenance of intracellular pH over a narrow range is crucial both for protein stability and for optimal enzyme catalysis rates (Somero, 1986). Hair cells may be particularly vulnerable to disturbances in acid/base status due to disruption of blood flow to the inner ear, because the main blood supply is concentrated in the lateral wall tissues, several tens of micrometers away from the hair cells. In addition, pH in the mammalian endolymph drops significantly during apnea (Ikeda et al., 1987) or anoxia (Bosher, 1979). Hair-cell shape and motility (Ikeda et al., 1992b) may be influenced by pH due to effects on cytoskeletal organization (Lamb et al., 1993). In addition, there may be an interplay between changes in intracellular pH and Ca$^{2+}$, either due to Na$^+$/H$^+$ and Na$^+$/Ca$^{2+}$ exchanger interactions, or due to the dependence of Ca$^{2+}$-calmodulin binding on pH (Busa and Nuccitelli, 1984). pH also may play a role in neuron excitability via effects on ion channel gating (Moody, 1984).

To date, the only studies on pH regulation in hair cells (Ikeda et al., 1991, 1992b; Ikeda et al., 1996), used cells with an unphysiologically high Na$^+$ concentration (> 100mM). In addition, there have been no studies on the interactions between regulation of intracellular pH and [Na$^+$], concentration in hair cells.

In most cell types, in a nominally HCO$_3^-$-free medium, the Na$^+$/H$^+$ exchanger is the major mechanism for removing acid equivalents during an acid load. Increased entry of Na$^+$ via the Na$^+$/H$^+$ exchanger during recovery from an acid load must be countered by the Na$^+$/K$^+$-ATPase in order to maintain a low [Na$^+$]. Activity of the Na$^+$/K$^+$-ATPase decreases outside a narrow pH range (Skou, 1957; Eaton et al., 1984) however, posing a
potential challenge to Na\(^+\) homeostasis. Thus we examined the effects of intracellular acid loading on both steady-state [Na\(^+\)], and Na\(^+\) entry rates in isolated goldfish hair cells.

We found that in the majority of hair cells, as in renal proximal tubule cells (Harris et al., 1986), intracellular Na\(^+\) concentration increases during recovery from an imposed intracellular acid load. In addition to the increase in Na\(^+\) entry via the Na\(^+\)/H\(^+\) exchanger, there may be a transient inhibition of the Na\(^+\),K\(^+\)-ATPase, as has been found in other intact cell preparations (Russell et al., 1983; Eaton et al., 1984; Harris et al., 1986) and was originally demonstrated biochemically (Skou, 1957).
METHODS AND MATERIALS

Solutions and chemicals

Unless otherwise noted, all solutions and chemicals were purchased from Sigma-Aldrich, Inc and the catalog numbers are given in parentheses. The procedures for the preparation of the solutions used in both hair cell dissociation and perfusion were developed and described in detail elsewhere (Mroz et al., 1993b). We briefly describe them below.

Solutions for cell dissociation. MEM (Minimum Essential Medium) Hepes (M2645) was adjusted to an osmolality of 250 mOsmol/kgH₂O by dissolving the powder for the 1 liter formulation into a final volume of 1.5 liters, buffering with Hepes/NaOH, and adding an extra 40mM NaCl. HBSS-M (H2387- Zero-Ca²⁺, Zero-Mg²⁺) was adjusted to an osmolality of 256 mOsm/kgH₂O by buffering with Hepes/NaOH (10mM Hepes, ~5mM NaOH) and diluting the final solution by 10% (in the petri dishes on the day of experiment). Both MEM and HBSS-M were adjusted to a pH of 7.5 with NaOH.

Solutions for perfusion. The composition of the Standard solution was as follows, in mM: 20 Heps, 100 NaCl, 3.5 KCl, 1 NaH₂PO₄, 0.5 Na₂SO₄, 0.6 MgCl₂, 1.2 CaCl₂, 5 D-Glucose, 4 L-Glutamine. MEM essential and non-essential amino acids were added at concentrations of the mammalian formulation and pH was adjusted to 7.5 with NaOH (~20 mM additional Na⁺). For the NH₄⁺ solution, 10mM Na⁺ was replaced by 10 mM
NH₄⁺. For the Zero-K⁺ solution, used to reversibly inhibit the Na⁺,K⁺-ATPase, KCl (3.5 mM) was replaced by NaCl.

Drugs

All stock solutions were stored in the dark. EIPA was made as a 100 mM stock in DMSO and stored at 8°C. Nigericin (14 mM stock in ethanol), and gramicidin (5 mM in DMSO) were stored at −20°C. BCECF and SBFI were prepared as 2 mM stocks in DMSO and stored desiccated.

Hair cell dissociation

Hair cells were prepared according to Protocol B from Mroz et al (Mroz et al., 1993b). Briefly, 8-18 cm long comet goldfish (Carrassius auratus) were chilled in water to near 0°C, then decapitated just behind the gills. The saccule, lagena, and utricle were removed from both sides and placed into a 25-mm petri dish containing HBSS-M. The tissues were cleaned and the lumenal sides of the sensory maculae exposed to the medium. The cleaned tissues were then transferred into HBSS-M containing 1 mM EGTA/3 mM NaOH for 20 minutes, followed by enzyme treatment in HBSS-M containing 0.625 mg/mL Collagenase D (Boehringer-Manheim #1 088 858) and 2.5 mg/mL Dispase (GibcoRL #17105-032) for 8 minutes. The tissues were then allowed to rest for 10 minutes in a petri dish containing 1.5 mL MEM supplemented with 0.5 mg/mL BSA (bovine serum albumin). The cells were released by mincing the tissue. The MEM solution containing the isolated cells was gently trituated at a volume of 0.3
mL 30 times using a Pipetman® (Rainin) with a 1 mL wide-bore pipette tip. Subsequently 450 µl of cells from the MEM petri dish were loaded with the fluorescent probe and the remaining cells in MEM were kept at 8°C for later use (up to about 6 hours).

*Loading of fluorescent ratio ion indicators*

Cells were loaded with either the membrane-permeant acetoxyethyl ester form of the Na⁺ indicator benzofuran isophthalate (SBFI-AM), or with the pH indicator BCECF-AM (both from Molecular Probes, Eugene, OR). DMSO was added to the vial containing 50 µg of the dye to make a 2 mM stock solution. For SBFI, equal volumes of the dye in DMSO and Pluronic F-127 20% w/v (Molecular Probes), a dispersing agent, were dissolved in MEM at a concentration of 100 µM and then mixed with 450 µl of cells in MEM inside a 1.5mL Eppendorf tube to give a final concentration of 10 µM SBFI (1% DMSO and Pluron F-127). For BCECF, the final concentration was 1 µM (0.1 % DMSO). The cells were then transferred to a coverglass-bottomed, open, laminar-flow, perfusion chamber (Warner instruments, Hamden, CT). The coverglass was pretreated with a 50 µL drop of 1 mg/ml concanavalin A (Sigma C2010) at 37°C for 1 hour and extensively washed with de-ionized water and MEM. The perfusion chamber was placed in a humidified petri dish and the cells were allowed to load with the fluorescent probe at room temperature in the dark. The loading time was 60 minutes for SBFI and 30 minutes for BCECF.
Measurements of intracellular Na\(^+\) or pH

Details of the fluorescence ratio ion imaging system are given elsewhere (Ronan, 2003). Briefly, the perfusion chamber was mounted on the stage of an inverted Zeiss 405M epi-fluorescence microscope. Cells were perfused at a rate of 500 μL/min, with a dual-head peristaltic pump. All experiments were carried out at room temperature. The cells were initially perfused with the Standard solution for at least 20 minutes, to wash away the dye and allow continued hydrolysis of any AM ester left inside the cells.

ISee analytical image processing software for Linux (Isee Imaging Systems, Raleigh, NC) was used to control the excitation filter wheel, shutters, emission filter wheel, and the CCD camera. For SBFI, cells were excited successively at wavelengths of 340 nm (Na\(^+\)-insensitive wavelength) and 380 nM (Na\(^+\)-sensitive) and emission was captured at 523 nM. For BCECF, cells were excited at 450 nM (pH insensitive) and 490 nM (pH sensitive), and emission collected at 535 nM. A 20X/0.75 NA Nikon Fluar objective lens was used for both phase contrast and epi-fluorescence imaging. Each pair of images was collected once every 30 or 60 seconds (~5s between each excitation wavelength). Both the neutral density filter and the exposure time of the excitation were adjusted to minimize the intensity of light exposure and to give an emission at least 3-6 times the background signal. The ratio of the emission from the two different excitation wavelengths (i.e. F340/F380 for SBFI, F490/F450 for BCECF) in a small region of interest (ROI) inside the cell was monitored online and the individual background-subtracted and ratio values (after subtracting the background, measured in a small ROI adjacent to the cell) were also saved in a table to be analyzed later.
Calibration

At the end of each experiment, an in situ calibration was performed for either intracellular Na\(^+\) concentration or pH. During calibration, a separate perfusion line was used to avoid contaminating the regular perfusion lines with ionophores (Bevensee et al., 1999). A manifold (Warner instruments, Hamden CT) was used to select between the regular solution line (from the peristaltic pump) and the calibration solution line (gravity fed), and the small (1.3-cm) piece of tubing between the manifold and perfusion chamber was replaced after each calibration.

**SBFI/Na\(^+\) calibration.** The pore-forming antibiotic gramicidin was perfused (5 \(\mu M\)) along with 3-5 solutions of differing Na\(^+\) concentrations. These calibration solutions were made by combining different volumes of equimolar high K\(^+\), low Na\(^+\) and high Na\(^+\), zero K\(^+\) solutions; Cl\(^-\) was replaced by gluconate to provide a concentration close to [Cl\(^-\)]\(_i\) (see Table 1 from (Ronan, 2003). A range of extracellular Na\(^+\) concentration from 2 mM up to 100 mM was used for the calibration. In most cases, a linear regression between the fluorescence ratio and each extracellular Na\(^+\) concentration provided a reliable calibration curve. Occasionally a non-linear hyperbolic fit was required, especially at higher Na\(^+\) concentrations (i.e. > 60mM), where the curve of ratio versus [Na\(^+\)]\(_i\) sometimes started to saturate. We performed at least a 3-point Na\(^+\) calibration in each cell at Na\(^+\) concentrations up to 60mM.

**BCECF/pH calibration.** The ionophore nigericin (10 \(\mu M\)) along with a high K\(^+\)-gluconate solution were used to clamp intracellular pH to extracellular pH. 3 different
pH solutions were used in the range 6.5 to 7.8; nigericin was only applied with the first calibration solution, at pH 7.3. A linear regression of the fluorescence ratio on pH provided calibration curves.

**pH sensitivity of SBFI.** Although BCECF fluorescence is little affected by changes in \([Na^+]_i\), some investigators have reported that the fluorescence of SBFI loaded cells is sensitive to changes in intracellular pH (Negulescu et al., 1990; Rose and Ransom, 1997a). We therefore examined the effect of clamping intracellular pH on cells during SBFI/Na\(^{+}\) calibration. We clamped intracellular Na\(^{+}\) concentration to 20 mM, using gramicidin as described above, in a solution with a normal extracellular pH of 7.4. We next perfused 10 \(\mu\)M nigericin to clamp intracellular pH to 7.4. Subsequently intracellular pH was clamped down to 6.8, at the same Na\(^{+}\) concentration. A pH\(_{i}\) drop of \(\sim 0.6\) units gave an apparent intracellular Na\(^{+}\) concentration decrease of \(12 \pm 2\) mM (n=5). Despite this effect of pH changes on SBFI ratio in Na\(^{+}\)- and pH-clamped cells, during experiments we never observed a drop in SBFI ratio during or after an acid load, only an increase.

*Rate of proton extrusion via the Na/H exchanger*

The rate of proton extrusion via the Na/H exchanger, \(\Phi_{Na/H}\) (mM/min), can be calculated from the initial rate of change in intracellular pH, \(dpH_i/dt\) (pH/min, linear fit to the first 5 data points) and the intracellular pH buffering capacity, \(\beta\) (pH\(_{i}\)) (mM/pH), at the pH\(_{i}\) just before the acid load, according to the following equation:

\[
\Phi_{Na/H} = \beta \times (pH_i) \times (dpH_i/dt)
\]
The dependence of the buffering capacity on intracellular pH$_i$ was previously determined (see Fig. 8 from (Ronan, 2003) to be linear with the following equation:

$$\beta (\text{pH}_i) = -52 \cdot \text{pH}_i + 403$$

**Steady-state Na$^+$ entry rate**

When the cell is in a steady-state, [Na$^+$]$_i$ is constant because the passive influx of Na$^+$ is balanced by active efflux via the Na$^+$,K$^+$-ATPase. We therefore measured the steady-state Na$^+$ entry rate as the initial rate of increase in [Na$^+$]$_i$ immediately after inhibition of the Na$^+$,K$^+$-ATPase (Abraham et al., 1985; Cohen and Lechene, 1989; Mroz et al., 1993a; Rose and Ransom, 1997a; Despa et al., 2002). We reversibly inhibited the pump by perfusion of the normal Standard solution without potassium ion (Zero-K$^+$ solution). We performed a linear regression of [Na$^+$]$_i$ versus time after immediately inhibition of the pump. The slope of this line (i.e. d[Na$^+$]$_i$/dt) gives the initial change in Na$^+$ versus time in units of mM/min (mmoles/(Liter*time)).

**Statistics**

Results are presented as means ± SEM (standard error of the mean) and statistical significance was tested using either paired (for repetitions within a cell) or unpaired Student’s t-tests (significance defined as p < 0.05).
RESULTS

\textit{pH}$_i$ response to acid loading

We measured intracellular pH$_i$ during an imposed intracellular acid load using the ammonium ion (NH$_4^+$) pre-pulse technique (Boron and De Weer, 1976). Figure 1A shows a typical pH$_i$ response to a 10 mM NH$_4^+$ pre-pulse and washout experiment, repeated twice in one cell. During the perfusion, ammonia gas (NH$_3$), in equilibrium with extracellular NH$_4^+$ ions, permeates the cell membrane and causes an initial alkalization. In 34 cells, pH$_i$ alkalinized from a control value of 7.35 $\pm$ .04 to 7.82 $\pm$ .04 during the NH$_4^+$ perfusion, followed by a slow relaxation to 7.4 $\pm$ .03 (n=25). Upon subsequent perfusion with ammonia-free standard solution, intracellular NH$_4^+$ ions rapidly dissociate into the membrane-permeant NH$_3$ gas, which exits the cell. The conversion of NH$_4^+$ to NH$_3$ produced a large intracellular acidification to pH 6.76 $\pm$ .04 (0.59 $\pm$ .04 pH drop from the resting level, n=34). The example cell (Fig. 1A) recovered rapidly from both imposed acid loads.

Recovery of intracellular pH following the acidification required the Na$^+$/H$^+$ exchanger since 10 $\mu$M EIPA, a specific blocker of the Na$^+$/H$^+$ exchanger, completely abolished the recovery (Fig 1B). Based on acid loading recovery experiments in which cells recovered by at least 0.3 pH units (22 out of 34 cells), the initial pH recovery rate was 0.21 $\pm$ .02 pH/min (n=22). To convert this pH recovery rate to proton extrusion rate via the Na$^+$/H$^+$ exchanger, we multiplied the pH$_i$-recovery rate by the buffering capacity at the acidic intracellular pH just before recovery (at a pH$_i$ of 6.76, $\beta$ (pH$_i$) = 52mM/pH, see Methods). The initial acid extrusion rate during recovery via the Na$^+$/H$^+$ exchanger,
determined this way, was 10.9 ± 1 mM/min, or 5x the steady-state rate of the exchanger in these cells (Ronan, 2003).

\[ [\text{Na}^+]_i \text{ response to acid loading: No exogenous pump inhibition} \]

Since the rate of the \( \text{Na}^+/\text{H}^+ \) exchanger increased dramatically in hair cells during an acid load, we decided to investigate whether intracellular \( \text{Na}^+ \) concentration also increased during the same type of perturbation. The steady-state \( \text{Na}^+ \) entry rate was first measured during a Zero-\( K^+ \) pump inhibition (Fig2 a,b,c). Subsequently, after recovery from the pump inhibition, the cell was acid loaded by a 10mM NH\(_4^+\) pre-pulse. In 13 out of 17 cells, \( \text{Na}^+ \) concentration increased substantially after acid loading, even though the \( \text{Na}^+,\text{K}^+-\text{ATPase} \) was not exogenously inhibited (Fig 2a,b). Of these cells, 8 recovered back towards control \( [\text{Na}^+]_i \) (Fig 2a) and 5 did not (Fig 2b). In the other 4 cells, the changes in \( [\text{Na}^+]_i \) in response to the acid load were too small to measure an initial rate (Fig 2c), either indicating that the \( \text{Na}^+ \)-pump was able to keep up with the increased \( \text{Na}^+ \) entry, or that the effect of pH\(_i\) on SBF\(_I\) ratio masked the increase in \( [\text{Na}^+]_i \) (see Methods).

In the example in Figure 2a, the steady-state \( \text{Na}^+ \) entry rate, measured during a Zero-\( K^+ \) \( \text{Na}^+ \)-pump inhibition, was 16 mM/min. Subsequently, during recovery from the acid load (NH\(_4^+\) washout without exogenous \( \text{Na}^+ \)-pump inhibition), the \( [\text{Na}^+]_i \) increased at an initial rate of 15 mM/min, causing \( [\text{Na}^+]_i \) to triple before recovering back towards control. The average initial rate of increase in \( [\text{Na}^+]_i \) during an acid load was 15.9 ± 3.8 mM/min (\( n = 8 \), including only cells which subsequently recovered to control \( [\text{Na}^+]_i \)). This rate of increase in \( [\text{Na}^+]_i \) represents a lower bound on the rate of \( \text{Na}^+ \) entry via the \( \text{Na}^+/\text{H}^+ \) exchanger, since small increases in \( [\text{Na}^+]_i \) should activate the \( \text{Na}^+,\text{K}^+-\text{ATPase} \). In
the same cells, the total steady-state Na\(^+\) entry rate was 12.6 ± 1.5 mM/min, thus the estimated rate of Na\(^+\) entry via the Na\(^+\)/H\(^+\) exchanger during an acid load (15.9 mM/min) was more than the total control steady-state Na\(^+\) entry rate.

Figure 3a shows the relationship between the initial rate of increase in [Na\(^+\)]\(i\) during the acid load and the steady-state Na\(^+\) entry rate, measured in the same cells (i.e. experiments like that shown in Figure 2a). For some of these cells the rate of increase in [Na\(^+\)]\(i\) after the acid load was much greater than the steady-state Na\(^+\) entry rate. Figure 3b shows the relationship between the peak [Na\(^+\)]\(i\) during recovery from an acid load and the control value. On average, the [Na\(^+\)]\(i\) reached a value almost twice the steady-state concentration before recovering back towards control.

From these experiments, we know that in most cells [Na\(^+\)]\(i\) increased during recovery from an acid load, but we could only estimate the additional Na\(^+\) entry rate. We directly measure this value in the next section.

**Na\(^+\) response to simultaneous pump inhibition and acid load**

In order to measure directly the initial Na\(^+\) entry rate during an acid load, we measured [Na\(^+\)]\(i\) during simultaneous acid load and pump inhibition (NH\(_4^+\) prepulse with Zero-K\(^+\)). Figure 4 shows one cell’s response to 3 different perturbations: an acid load during pump inhibition (NH\(_4^+\) washout + Zero-K\(^+\)), an acid load without pump inhibition (NH\(_4^+\) washout alone), and pump inhibition alone (Zero-K\(^+\)). The initial rate of Na\(^+\) entry during simultaneous acid load and pump inhibition was double the steady-state entry rate (12.6 mM/min ± 1.2 SEM steady state versus 25.3 mM/min ± 2 SEM during NH\(_4^+\) washout, paired difference of 12.7 ± 2.3 mM/min, n = 3, P < .05). Despite the large
increase in Na⁺ influx during the simultaneous acid load and Na⁺-pump inhibition, the cells were all able to recover back to the control [Na⁺], after removal of inhibition.

If we assume that the increase in Na⁺ entry rate during an acid load, above the steady-state entry, was due solely to an increased Na⁺ influx via the Na⁺/H⁺ exchanger, then the Na⁺ entry via the Na⁺/H⁺ exchanger was 12.7 ± 2.3 mM/min (the mean paired difference between the two conditions, n = 3) during recovery from an acid load. This is similar to the initial increase in [Na⁺], during an acid load without exogenous pump inhibition (15.9 mM/min). In addition this Na⁺ entry rate is similar to the acid equivalent extrusion rate, estimated from the initial rate of recovery of intracellular pHᵢ, of 10.9 mM/min. Hence three methods of measuring the rate of the Na⁺/H⁺ exchanger during an acid load, extrusion rate of acid equivalents (from pHᵢ measurements), Na⁺ entry rates (from [Na⁺], measurements during Na⁺-pump inhibition), and increases in [Na⁺], without exogenous pump inhibition gave virtually the same results.
DISCUSSION

This study examined tradeoffs in Na\(^+\) and pH regulation in goldfish hair cells. pH\(_i\) may influence intracellular [Na\(^+\)]\(_i\) in two ways: increased Na\(^+\) influx via the Na\(^+\)/H\(^+\) exchanger and decreased Na\(^+\) efflux via inhibition of the Na\(^+\),K\(^+\)-ATPase. Such an effect of pH\(_i\) on [Na\(^+\)]\(_i\) may be a particular issue in cells that release neurotransmitter. For example, in hippocampal neurons, intracellular acidification caused an increase in spontaneous neurotransmitter release (Trudeau et al., 1999). The authors found that during recovery from the acidification, increased Na\(^+\) influx via the Na\(^+\)/H\(^+\) exchanger elevated [Na\(^+\)]\(_i\), which in turn increased [Ca\(^{2+}\)]\(_i\) via a reverse operation of the Na\(^+\)/Ca\(^{2+}\) exchanger. Although they did not investigate the issue, the elevation in [Na\(^+\)]\(_i\) may also have been due to the acidification inhibiting the Na\(^+\),K\(^+\)-ATPase. In hair cells, intracellular pH changes might also influence both resting and sound evoked levels of neurotransmitter release. Such interactions between intracellular acidification, the Na\(^+\)/H\(^+\) exchanger, the Na\(^+\)/Ca\(^{2+}\) exchanger, and the Na\(^+\),K\(^+\)-ATPase may also be a crucial component of damage to cells in various models of anoxia or ischemia/reperfusion injury (Piper et al., 1998), although this issue has not directly been examined in cochlear ischemia models (Ren et al., 1995; Mom et al., 1997).

We discuss three main issues below. First we compare the steady-state and acid-loaded rates of the Na\(^+\)/H\(^+\) exchanger among different cell types. We next compare our responses in hair cells to other cell types in which [Na\(^+\)]\(_i\) was measured during/after an intracellular acid load. Finally we discuss whether the Na\(^+\),K\(^+\)-ATPase in hair cells is inhibited by an acid load.
Comparison of steady-state and acid-loaded rates of the Na\(^+\)/H\(^+\) exchanger among different cell types

In many cell types, the Na\(^+\)/H\(^+\) exchanger is quiescent at resting pH\(_i\) (Grinstein and Rothstein, 1986) and switches on rapidly during an intracellular acidification, due to an allosteric H\(^+\) modifier site (Aronson et al., 1982). Table 1 lists examples of Na\(^+\)/H\(^+\) rates in the steady state and after an acid load in various cell types. Red blood cells, thymic lymphocytes, guinea-pig outer hair cells (Table 1), and lung fibroblasts (Paris and Pouyssegur, 1984) all have Na\(^+\)/H\(^+\) exchangers which are inactive at the resting pH\(_i\) but which can be activated during an acid load. Goldfish hair cells, on the other hand, do have resting activity of the Na\(^+\)/H\(^+\) exchanger (~2 mM/min), which accounts for 1/5 of the total steady-state Na\(^+\) entry (Ronan, 2003).

In this study we found that the rate of Na\(^+\) entry via the Na\(^+\)/H\(^+\) exchanger increased by a factor of 6, to 12 mM/min, during recovery from an acid load. The steady-state and acid-loaded rates of the Na\(^+\)/H\(^+\) exchanger in goldfish hair cells were very similar to those of renal proximal tubule cells (Table 1), which are absorptive epithelial cells involved in rapid ion transport (Lechene, 1988), and an order of magnitude faster than in goldfish hepatocytes (Table 1). Goldfish hair cells were very similar to other excitable cells (neurons, skeletal muscle, and cardiac myocytes) and cells involved in rapid ion transport (renal proximal tubule cells) both in terms of resting and acid-loaded rates of the Na\(^+\)/H\(^+\) exchanger.
Other cell types also exhibit increases in $[Na^+]_i$ during recovery from an acid load

In the majority of hair cells, $[Na^+]_i$ doubled during recovery from an intracellular acid load (Fig. 3b). These increases could not be attributed to the pH-sensitivity of SBFI, since the effect was in the opposite direction (see Methods). Thus, if anything, the increases in Na$^+$ concentration we measured during recovery from an acid load might have been an underestimate. Several other studies have shown that intracellular acidification can increase $[Na^+]_i$. For example, in sublingual mucous acinar cells, $[Na^+]_i$ concentration doubled during a 0.2 pH unit acidification, imposed with sodium-propionate (Zhang et al., 1993). In renal proximal tubule cells, acidified by an NH$_4^+$ prepulse, $[Na^+]_i$ tripled 60 seconds after the acid load (Harris et al., 1986).

In other cell types, intracellular acid loading caused either negligible changes in $[Na^+]_i$ or even decreases. For example, in sheep cardiac Purkinje fibers intracellular $[Na^+]_i$ barely changed either during application or withdrawal of NH$_4^+$ (Deitmer and Ellis, 1980), implying that the Na$^+$-pump was able to deal with the increased Na$^+$ load for these cells. Interestingly, in frog skin epithelium, acidification caused a decrease in $[Na^+]_i$ due a reduction in the apical Na$^+$ conductance (Ehrenfeld et al., 1992). In 4 of 17 goldfish hair cells we saw only small changes in $[Na^+]_i$ (Fig 2c) during recovery from an acid load, but in these cells an increase in $[Na^+]_i$ may have been masked by the effect of pH on SBFI ratio (see Methods).
Does intracellular acidification inhibit the Na\(^+\),K\(^+\)-ATPase?

Figure 5 summarizes steady-state and acid-loaded rates of the Na\(^+\)/H\(^+\) exchanger, total Na\(^+\) entry (during exogenous Na\(^+\)-pump inhibition), and [Na\(^+\)]\(_i\) (no exogenous Na\(^+\)-pump inhibition). The rate of extrusion of acid equivalents via the Na\(^+\)/H\(^+\) exchanger during an acid load, measured from changes in intracellular pH, was 10.9 mM/min. The rate of Na\(^+\) entry via the Na\(^+\)/H\(^+\) exchanger, measured as the difference between the total Na\(^+\) entry rate during an acid load versus the steady-state Na\(^+\) entry rate, was 12.7 mM/min, not different from the rate of extrusion of acid equivalents. Hence both methods give similar measures of the acid-loaded rate of the Na\(^+\)/H\(^+\) exchanger, and agree with the stoichiometry of 1:1 for the exchanger.

During recovery from an intracellular acid load, unless the Na\(^+\) extrusion rate via the Na\(^+\),K\(^+\)-ATPase drops below its steady-state level, we would thus expect intracellular Na\(^+\) concentration to increase at a rate no higher than 12.7 mM/min. Any net activation of the Na\(^+\),K\(^+\)-pump by the higher [Na\(^+\)]\(_i\) (Skou, 1957; Cohen and Lechene, 1989) would lead to an even lower rate of increase in [Na\(^+\)]\(_i\). For example, the Na\(^+\),K\(^+\)-ATPase in goldfish hair cells has the capacity to at least double its rate in response to increases in intracellular Na\(^+\) concentration (Mroz et al., 1993a; Ronan, 2003). The observed initial rate of increase in [Na\(^+\)]\(_i\) was 15.9 mM/min, suggesting that the Na\(^+\),K\(^+\)-pump was not activated by the increases in [Na\(^+\)]\(_i\) during recovery from an acid load. In addition, half of the hair cells (Fig 4a) had an initial rate of increase of [Na\(^+\)]\(_i\) higher than the expected
Na⁺ entry rate via the Na⁺/H⁺ exchanger (12.7 mM/min). For these cells, the Na⁺,K⁺-ATPase was most likely inhibited due to the acidic intracellular pH.

Several other studies have directly shown that intracellular acidification inhibits the Na⁺,K⁺-ATPase (Skou, 1957; Russell et al., 1983; Eaton et al., 1984; Harris et al., 1986). In renal proximal tubule cells, which have a large percentage (80 %) of steady-state Na⁺ entry via Na⁺/H⁺ exchanger, Na⁺ influx increased by a factor of 6, [Na⁺]i, increased by a factor of 3, and the Na⁺,K⁺-ATPase was initially inhibited by 50% during the first 60 seconds after an acid load (Harris et al., 1986). Also in barnacle muscle fibers, Na⁺ efflux was completely absent for pHₐ < 6.8 (Russell et al., 1983). Although we did not directly measure the rate of the Na⁺,K⁺-ATPase during the acid load, our results are also consistent with the idea that acid loading, in addition to increasing Na⁺ entry via the Na⁺/H⁺ exchanger, may also inhibit the Na⁺-pump. Figure 2b shows an extreme example in which the Na⁺-pump must have been completely inhibited since [Na⁺]i increased dramatically (> 70 mM) after an acid load and remained elevated.

We also have seen that in some hair cells, which were not able to recover from an exogenously applied Na⁺-pump inhibition, the intracellular alkalinization during NH₄⁺ addition caused decreases in [Na⁺]i back towards the control levels (data not shown). The implication is that in these cells, the Na⁺,K⁺-ATPase may have been inhibited by an acidic intracellular pH, which was subsequently relieved by the alkalinization.

Rapid correction of intracellular pH is vital to many cellular processes. Elevation of intracellular Na⁺ concentration, on the other hand, may be more easily tolerated. For example, in human diploid fibroblasts, inhibition of the Na⁺,K⁺-ATPase for several hours led to large increases in intracellular Na⁺ concentration with little effect on cellular
volume and which were rapidly corrected upon re-activation of the pump (Abraham et al., 1985). Thus during an intracellular acid load there may be a temporary trade-off of Na\(^+\) regulation in favor of pH regulation.
CAPTIONS, TABLES, AND FIGURES

Table 1: Steady-state and acid loaded rates of the Na⁺/H⁺ exchanger (mM/min) in various cell types. For comparison with this study, the acid-loaded rates were taken as the rate 0.6 units more acid than the resting value. In all of these cells, experiments were carried out in nominally HCO₃⁻- free media, and acid loads were produced either using an NH₄⁺ pre-pulse or propionic acid. The rows are sorted from lowest to highest acid-loaded Na⁺/H⁺ rate, showing that goldfish hair cells have one of the highest rates.

Figure 1. (A.) Response of intracellular pH to an NH₄⁺ pulse, repeated twice in one cell. During perfusion of 10 mM NH₄⁺ the cell rapidly alkalinizes, followed by a slow relaxation. Washout of NH₄⁺ causes a large and rapid acidification, followed by recovery. (B.) Response of intracellular pH to an NH₄⁺ pulse and washout, followed by a second NH₄⁺ pulse in which washout was accompanied by 10uM EIPA. The EIPA, which is a specific inhibitor of the Na⁺/H⁺ exchanger, completely inhibited the recovery from the second acid load.

Figure 2: [Na⁺], during Na⁺-pump inhibition and recovery using Zero-K⁺, followed by a 10 mM NH₄Cl pulse and washout to cause an intracellular acid load. (A.) Note the large increase in [Na⁺], concentration imposed by NH₄⁺ washout (rate of 15 mM/min), even though the pump was not exogenously inhibited, followed by recovery back to control. The control steady-state rate of Na⁺ entry, measured during the Zero-K⁺ Na⁺-pump inhibition, was 16 mM/min for this cell. (B.) This cell showed a dramatic increase in
[Na\(^+\)]\textsubscript{i} after an acid load and did not recover. (C.) In this cell [Na\(^+\)]\textsubscript{i} did not appear to change after the acid load.

Figure 3: Summary of experiments in which the Na\(^+\),K\(^-\)-ATPase was inhibited and allowed to recover, followed by an acid load and recovery in the same cell in each of 8 cells. (A.) The initial net rate of increase of [Na\(^+\)] \textit{during recovery} from an acid load (no exogenous Na\(^+\)-pump inhibition) is plotted versus the control steady-state Na\(^+\) entry rate during Na\(^+\),K\(^-\)-ATPase inhibition for each of these cells. (B.) The peak [Na\(^+\)]\textsubscript{i} during an acid load is plotted versus the control [Na\(^+\)]\textsubscript{i} before the acid load. Based on the linear fit, the [Na\(^+\)]\textsubscript{i} increased by a factor of 1.7 during an acid load.

Figure 4: [Na\(^+\)]\textsubscript{i} response to simultaneous acid load and Zero-K\(^+\) Na\(^+\)-pump inhibition. First the Na\(^+\)-pump was inhibited during an acid load (NH\(_4\)\(^+\) washout + Zero-K\(^+\)) and the initial rate of Na\(^+\) entry was 25 mM/min. Next, the cell was also acid loaded without pump inhibition and showed a slight increase (initial rate \textit{\sim} 10 mM/min) followed by recovery. Finally, the Na\(^+\)-pump was inhibited (Zero-K\(^+\) alone) and the control steady-state rate of Na\(^+\) entry was 15 mM/min. Note that the difference between the Na\(^+\) entry rate (measured by Na\(^+\)-pump inhibition) during an acid load (first treatment) and control (last treatment) matches the initial rate of increase in [Na\(^+\)]\textsubscript{i} during the acid load (middle treatment).

Figure 5: Summary of rates measured at rest and during/after an imposed intracellular acid load. The steady-state Na\(^+\)/H\(^+\) rate was measured in a previous study and reproduced
here for comparison (Ronan, 2003). The acid-loaded rate of the Na⁺/H⁺ exchanger was measured as the initial rate of increase in intracellular pH (dpH/dt in pH/min) multiplied by the intracellular pH buffering capacity (β) during recovery from the acid load. The steady-state total Na⁺ entry rates were measured as the initial d[Na⁺]/dt during inhibition of the Na⁺,K⁺-ATPase with Zero-K⁺. The acid-loaded total Na⁺ entry rate was measured as the initial d[Na⁺]/dt during simultaneous acid load and inhibition of the Na⁺,K⁺-ATPase with Zero-K⁺.
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<th>Cell Type</th>
<th>Reference</th>
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<th>Resting Na^+/H^+ (mM/min)</th>
<th>Na^+/H^+ acid load (mM/min)</th>
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Table 1
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
CHAPTER 5: Conclusion and Implications

CONCLUSION

This study was an integrated approach to understanding hair cell function. Other studies on hair cells have characterized individual ion conductances using open-loop, patch-clamp techniques. We measured Na\(^+\) entry pathways and regulatory capabilities in cells which were able to respond to and recover from perturbations using their native mechanisms of ion homeostasis. We uncovered processes that occur in the resting state of the hair cells yet are almost impossible to study with an electrophysiological approach.

Since Na\(^+\)-coupled transport is used to energize almost all ion and metabolite transport across the plasma membrane, the steady-state Na\(^+\) entry rate is a measure of the cell’s basic energy demands. We showed that isolated goldfish hair cells have high steady-state rates of Na\(^+\) entry, compared to many other cell types, which most likely represent the in vivo steady-state Na\(^+\) load.

In addition, the major pathways of resting Na\(^+\) entry describe the differentiated functions of the cell (Lechene, 1988). In our study we found that isolated goldfish hair cells have significant components of Na\(^+\) entry via transduction channels, the Na\(^+\)/H\(^+\) exchanger, and the Na\(^+\)/Ca\(^{2+}\)-(K\(^+\)) exchanger, in addition to a K\(^+\)-depolarization-sensitive component. The purpose of the Na\(^+\)/H\(^+\) component may be to counter a resting acid permeability in the plasma membrane. The Na\(^+\)/Ca\(^{2+}\)-(K\(^+\)) exchanger is involved in balancing a steady-state influx of calcium, a portion of which enters via L-type calcium
channels. The resting calcium influx is most likely involved in spontaneous neurotransmitter release. Goldfish hair cells were similar to cardiac myocytes in having significant steady-state Na⁺ entry via both Na⁺/H⁺ and Na⁺/Ca²⁺-(K⁺) exchangers, although they were dissimilar in not having a significant TTX-sensitive component (Despa et al., 2002).

We further characterized the Na⁺/H⁺ exchanger in response to acid loading and showed that not only does Na⁺ entry via the exchanger increase six-fold, but the Na⁺,K⁺-ATPase is also initially inhibited by the intracellular acidification, posing a potential conflict to maintenance of Na⁺ homeostasis.

**IMPLICATIONS**

Maintenance of intracellular Na⁺ homeostasis is crucial both for the long-term health of hair cells, and for their function as mechanical-electrical transducers. Hair cells are highly active cells even at rest, with a constant depolarizing cation influx via the transduction channels resulting in basolateral Ca²⁺ influx and subsequent neurotransmitter release. Hair cells must be able to sense small changes in membrane potential during stimulation. The exquisite sensitivity and timing of the cells’ rapid responses to sound therefore depend on the slower background processes of ion homeostasis to maintain the steady-state operating point of the cell.

In addition, various perturbations, including acoustic stimulation (Saunders et al., 1985), ototoxic drugs, and changes in cochlear microcirculation (Ikeda et al., 1994), can result in either temporary or permanent damage to hair cells. The vulnerability of hair cells to physiological perturbations, regardless of the initial mechanism of insult,
ultimately results in a breakdown of ion homeostasis. For example, in alligator lizard cochlea, noise-induced temporary threshold shift (i.e. a temporary decrease in the cell’s sensitivity to sound) is associated with changes in hair cell volume (Dew et al., 1993). In the mammalian cochlea, subtle anatomical correlates of sound-induced threshold shifts, such as a slight shortening of the supracuticular rootlets of the stereociliary bundle, and swelling or vacuolization of the inner hair cells (Liberman and Dodds, 1987), also might result from disruption in the normal processes of ion balance.

Knowledge of the boundaries of Na\(^+\) ion homeostasis in hair cells, as well as the main transport processes involved in Na\(^+\) entry, has implications for prevention of hair cell degeneration. For example, a component of ischemia/perfusion injury in the heart is due to intracellular acidosis (Katsura et al., 1992; Piper et al., 1998), followed by an elevation of intracellular Na\(^+\) from activation of the Na\(^+/\)H\(^+\) exchanger (Lazdunski et al., 1985). The increased [Na\(^+\)], drives the reverse operation of the Na\(^+/\)Ca\(^{2+}\) exchanger, causing Ca\(^{2+}\) overload and subsequent processes leading to cell degeneration (Duchen, 2000). Application of drugs to block the Na\(^+/\)H\(^+\) exchanger during ischemia/reperfusion have shown some promise in prevention of injury in the heart (Avkiran and Marber, 2002). The same types of treatment may prevent ischemic damage to hair cells, such as might occur during surgeries which temporarily disrupt the blood supply to the cochlea (removal of tumors on the auditory nerve). Indeed several forms of hearing loss may be due to a disruption of blood flow to the cochlea, including cases of sudden idiopathic hearing loss or presbycusis (Schuknecht, 1974). In the brain, conditioning via a short, reversible ischemic exposure has been shown to decrease the extent of injury during a subsequent longer exposure to ischemia by reducing the increases in [Ca\(^{2+}\)]\(_i\) (Shimazaki
et al., 1998). The added protection may have been due to an increase in the activity of calcium homeostatic processes, such as the Na\(^+\)/Ca\(^{2+}\) exchanger. A similar paradigm may exist for the effects of sound (Niu and Canlon, 2002) or heat-shock (Yoshida et al., 1999) conditioning on prevention of hair cell damage after acoustic trauma. In addition, development of drugs to increase the long-term expression of Na\(^+\),K\(^+\)-ATPase in mammalian hair cells might make these cells more robust during times of intense acoustic stimulation.
References Cited


