Effects of Strontium on the Permeation and Gating Phenotype of Calcium Channels in Hair Cells

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Rodriguez-Contreras A, Lv P, Zhu J, Kim HJ, Yamoah EN. Effects of strontium on the permeation and gating phenotype of calcium channels in hair cells. J Neurophysiol 100: 2115–2124, 2008. First published August 13, 2008; doi:10.1152/jn.90473.2008. To minimize the effects of Ca2+-buffering and signaling, this study sought to examine single Ca2+-channel properties using Sr2+-ions, which substitute well for Ca2+ but bind weakly to intracellular Ca2+-buffers. Two single-channel fluctuations were distinguished by their sensitivity to dihydropyridine agonist (L-type) and insensitivity toward dihydropyridine antagonist (non-L-type). The L- and non-L-type single channels were observed with single-channel conductances of 16 and 19 pS at 70 mM Sr2+, respectively. We obtained Kd estimates of 5.2 and 1.9 mM for Sr2+ for L- and non-L-type channels, respectively. At Ca2+-concentrations of ~2 mM, the single-channel conductances of Sr2+ for the L-type channel was ~1.5 and 4.0 pS for the non-L-type channels. Thus the limits of single-channel microdomain at the membrane potential of a hair cell (e.g., ~65 mV) for Sr2+ ranges from 800 to 2,000 ion/ms, assuming an E<sub>Ca</sub> of 100 mV. The channels are ≥4-fold more sensitive at the physiological concentration ranges than at concentrations >10 mM. Additionally, the channels have the propensity to dwell in the closed state at high concentrations of Sr2+, which is reflected in the time constant of the first latency distributions. It is concluded that the concentration of the permeant ion modulates the gating of hair cell Ca2+-channels. Finally, the closed state/s that is/are altered by high concentrations of Sr2+ may represent divalent ion-dependent inactivation of the L-type channel.

INTRODUCTION

Ca2+-channels are multi-ion pores that allow the passive flow of several divalent, and under restricted conditions, monovalent cations (Hess et al. 1986; Hille 2001). Among the permeant ions, Sr2+ by virtue of its similar charge and size to Ca2+, produced currents that are akin to Ca2+-currents (Bourenet et al. 1996; Byerly et al. 1985; McNaughton and Randall 1997). The magnitude and time constants of activation and deactivation of whole cell Sr2+-currents are similar to the properties of Ca2+-currents of Ca<sub>2.2</sub> channels. The properties of Sr2+-currents are not identical to Ca2+-currents, however, and the differences rely mainly on the magnitude and extend inactivation (McNaughton and Randall 1997). Moreover, the effects of Sr2+- on the elementary properties of Ca2+-channels remain uncertain. Instead, much attention has focused on studies of the effects of Ba2+- on the macroscopic and microscopic phenotype of Ca2+-channels, partly because Ba2+-currents are ≥2-fold larger than Ca2+-currents, and Ba2+-currents are moderately stable compared with the fast rundown of Ca2+-currents (Fox et al. 1987; Rodriguez-Contreras et al. 2002; Wakamori et al. 1998; Yue and Marban 1990). Ba2+-markedly alters not only the voltage- and time-dependent activation and deactivation kinetics but also abolishes inactivation in Ca2+-channels that rely on Ca2+ and calmodulin in conferring the decay of the current (Imredy and Yue 1994; Mori et al. 2004). Apparently, whereas Ba2+ is a poor substitute for Ca2+ in various biological processes, Sr2+ can replace Ca2+ in several Ca2+-dependent reactions (Falke et al. 1994; Xu-Friedman and Regehr 1999).

In addition to traversing Ca2+-channels, Sr2+-mediates neurotransmitter release in the squid giant synapse (Augustine and Eckert 1984), the neuromuscular junction (Bain and Quastel 1992; Dodge et al. 1969), hippocampal synapse (Abdul-Ghani et al. 1996), and cerebellar synapses (Falke et al. 1994; Xu-Friedman and Regehr 1999). Invariably, the effects of Sr2+-on neurotransmitter release are desynchronized and reduced compared with Ca2+, suggesting that Sr2+ is less effective than Ca2+ in triggering exocytotic transmitter release (Falke et al. 1994; Xu-Friedman and Regehr 1999).

Ohmori (1985) showed that, in vestibular hair cells of the chick, Sr2+-maintained mechanoelectrical transducer currents. In addition, activation of Ca2+-activated K+ channels (BK channels) can be supported by Sr2+ in goldfish saccular hair cells (Sugihara 1998). Because hair cells in lower vertebrates rely on the interplay between the activation of voltage-gated Ca2+-channels and the activation of BK channels to confer electrical tuning (Armstrong and Roberts 1998; Art et al. 1993; Fuchs and Evans 1988; Lewis and Hadspheth 1983), and Sr2+ can replace Ca2+ to induce membrane electrical resonance, we surmised that understanding the effects of Sr2+ on the elementary properties of Ca2+-channels in hair cells would provide clues to the mechanisms of hair cell functions. Indeed, to prevent cross-talk between different Ca2+-dependent pathways in hair cells, the macro-domain Ca2+ handling is tightly regulated by mobile Ca2+-buffers (Hall et al. 1997). Thus incoming Ca2+ at the micro-domains of Ca2+-channels is a major determinant of Ca2+-dependent functions. Additionally, because intracellular Ca2+-buffers bind weakly with Sr2+ (Yawo 1999), we rationalized that Sr2+ flux through Ca2+-channels represents an unadulterated means to study the throughput of the channel. Thus we determined the unitary current phenotypes of Ca2+-channels of bullfrog saccular hair cells using Sr2+ as the charge carrier.
METHODS

American bullfrogs (Rana Castelbeina) were purchased from West Jersey Biological Supply Co. (Wenonah, NJ) and Nebraska Scientific (Omaha, NB) and kept in the laboratory in freshwater circulating containers. The methods for dissociating single hair cells from the saccule of bullfrogs and for the set-up of patch-clamp experiments (Hamill et al. 1981) have been described previously (Rodriguez-Contreras and Yamoah 2001; Rodriguez-Contreras et al. 2002; Yamoah and Gillespie 1996). All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted. Bullfrogs were killed, and inner ears were removed and placed quickly in oxygenated, low Ca\(^{2+}\) solution (LCS; in mM): 110 NaCl, 2 KCl, 3 d-glucose, 0.1 CaCl\(_2\), and 5 HEPES, pH 7.4, with NaOH. To disrupt the macular tight junctions, the preparation was exposed to 4 mM EGTA for 15 min. After washing the preparation with fresh saline, the saccular macula was isolated and incubated in frog saline containing 50 μg/ml protease (type XXIV) for 20 min. The tissue was washed and transferred to frog saline containing 1 mg/ml bovine serum albumin (Sigma) and 2 mg/ml DNase I ( Worthington, Lakewood, NJ) for 10 min. This procedure has been used previously (Chabbert 1997) and differs from more severe enzymatic treatments that can affect ionic conductances (Armstrong and Roberts 1998). The preparation was washed and placed on the recording chamber at 4°C before recording at room temperature. The otolithic membrane was removed mechanically, and hair cells were dissociated from the macula using an eyelash. Hair cells were allowed to settle onto the bottom of the recording chamber (coated with the lectin concavalin-A) for 20 min before patch-clamp recording.

Patch pipettes were pulled from borosilicate and quartz glass capillaries (World Precision Instruments Sarasota, FL and Sutter Instruments, San Rafael, CA) on a Flaming-Brown and laser micro-electrode pullers (Sutter Instruments). For single-channel recordings, the borosilicate glass electrodes were coated with Sylgard 184 (Dow-Corning, Midland, MI) ≤100 μm from the tip and fire-polished before use. Bath solution contained (in mM) 90 NaCl, 25 tetraethyl-ammonium chloride (TEACl), 5 4-aminopyridine (4-AP), 5 BaCl\(_2/\)SrCl\(_2\), 3 glucose, and 5 HEPES (pH 7.4). Ca\(^{2+}\) currents were recorded using perforated patch electrodes (resistances 2–4 MΩ) whose tips contained (in mM) 120 CsCl and 5 HEPES (neutralized to pH 7.3 with CsOH). To gain electrical access to hair cells and to minimize wash-out of intracellular molecules, patch electrodes were backfilled with solution containing (in mM) 130 CsCl, 1 CaCl\(_2\), 5 HEPES, and amphotericin 200 μg/ml (neutralized to pH 7.3 with CsOH) (Korn et al. 1991). To ensure that recordings are made in the perforated-patch mode instead of whole cell mode, the backfilled solution of the patch electrode contained 1 mM Ca\(^{2+}\). A switch from the perforated to whole cell mode resulted in cell death caused by Ca\(^{2+}\) toxicity. Series resistance (5–10 MΩ) was compensated (nominally 50–60%). Liquid-junction potentials were not corrected because they were <2 mV. Voltage-clamped Ba\(^{2+}/\)Ca\(^{2+}/\)Sr\(^{2+}\) currents were amplified with Axopatch 200B amplifiers ( Molecular Devices, Sunnyvale, CA). Cell capacitance was calculated by integrating the area under an uncompensated capacitive-transient elicited by a 20-mV hyperpolarizing pulse from a holding potential of −80 mV. Cell capacitance and series resistance was compensated as much as possible almost to the point of ringing. In general, 60–80% of the series resistance was compensated. Current records were filtered at 2–5 kHz with a low-pass Bessel filter and digitized at 10 kHz with a Digidata interface controlled by custom-made software.

For single-channel recordings, patch electrodes were filled with a Sr\(^{2+}\) solution (5–70 mM) containing (in mM) 20 TEACl, 5 4-AP, and 5 HEPES at pH 7.4 (adjusted with TEAOH). N-methyl-d-glucamine (NMG) was used to substitute for divalent Sr\(^{2+}\) and to maintain an osmolarity of ~280 mmosmol. Quartz glass electrodes were used to record single-channel fluctuations when the Sr\(^{2+}\) concentration was <20 mM. To identify L-type channels, experiments were conducted in the presence of the channel’s agonist Bay K 8644 (Calbiochem, La Jolla, CA). Stock solutions of Bay K 8644 (100 mM) were made in DMSO, and a final concentration of 5 μM was used. The bath solution contained (in mM) 80 KCl, 3 d-glucose, 20 TEACl, 1 CaCl\(_2\), 5 4AP, and 5 HEPES, pH 7.4, with TEAOH, to shift the resting potential to ~0 mV (Rodriguez-Contreras and Yamoah 2003). Here, liquid junction potentials were measured and corrected as described previously (Rodriguez-Contreras et al. 2002). Experiments were carried out at room temperature (~21°C).

Data analysis

Whole cell Ba\(^{2+}/\)Ca\(^{2+}/\)Sr\(^{2+}\) current amplitudes at varying test potentials were measured at the peak and steady-state levels using a peak and steady-state detection routine. For single channel records, leakage and capacitative transient currents were subtracted by fitting a smooth template to null traces. Leak-subtracted current recordings were idealized using a half-height criterion. Transitions between closed and open levels were determined using a threshold detection algorithm, which required that two data points exist above the half mean amplitude of the single-unit opening. The computer-detected openings were confirmed by visual inspection, and sweeps with excessive noise were discarded. Amplitude histograms at a given test potential were generated and fitted to a single Gaussian distribution using a Levenberg-Marquardt algorithm to obtain the mean and SD. At least five voltage steps and their corresponding single-channel currents were used to determine the unitary conductance. Single-channel current-voltage relations were fitted by linear least-square regression lines and single-channel conductances were obtained from the slope of the regression lines. Idealized records were used to construct ensemble-averaged currents and open probability (P\(_o\)).

Additional kinetic analyses were performed on patches, which contained only one channel. The criteria consisted of quantitative determination followed by 10.220.33.1 on October 28, 2016 http://jn.physiology.org/ Downloaded from J Neurophysiol • VOL. 100 • OCTOBER 2008 • www.jn.org
by visual inspection of the data. The patches contained one channel, as there was no stacking of events. Furthermore, direct transition from fast to slow kinetics with similar current amplitude, and vice versa, were observed in the selected recordings, indicating that the gating modes were derived from a single channel. The cumulative first latency histograms were determined as described by (Zei and Aldrich 1998). Curve fits and data analyses were performed using Origin software (MicroCal). All averaged and normalized data are presented as means ± SE. Frogs were housed and killed using a protocol approved by the University of California, Davis, IACUC Committee.

RESULTS

We determined the similarities and differences in the magnitude and profile of whole cell Ba\(^{2+}\), Ca\(^{2+}\), and Sr\(^{2+}\) currents in hair cells. Whereas replacing Ca\(^{2+}\) with Ba\(^{2+}\) in the recording bath solution strongly affected the amplitude and decay of the whole cell inward currents in saccular hair cells, the effects of Sr\(^{2+}\) were relatively modest (Fig. 1). Inward currents were elicited from a holding potential of −60 mV and a step potential to −10 mV. Outward K\(^{+}\) currents were blocked with external 4-AP and TEA as well as internal Cs\(^{+}\). On switching the bath solution, which contained 5 mM Ca\(^{2+}\), to one that contained the same concentrations of Ba\(^{2+}\), the inward current increased by −1.4-fold. The mean amplitudes of the current (in pA) for Ca\(^{2+}\) and Ba\(^{2+}\) were 778 ± 56 and 992 ± 101 (n = 7; P < 0.01), respectively. Additionally, Ba\(^{2+}\) abolished the slow decay of the current profile. In contrast, in 5 mM Sr\(^{2+}\), the inward current mirrored roughly the Ca\(^{2+}\) current in magnitude, activation, and deactivation. The mean amplitudes of the current (in pA) for Sr\(^{2+}\) and Ca\(^{2+}\) were 755 ± 62 and 811 ± 45 (n = 7; P = 0.08), respectively. Inactivation kinetics differed slightly, which is shown in the inset in Fig. 1. Although some of the macroscopic features of Sr\(^{2+}\) and Ca\(^{2+}\) currents appeared similar, single-channel currents should confirm these parallels and reveal the distinct properties of Sr\(^{2+}\) currents.

Inward Ca\(^{2+}\) currents from hair cells in the saccule are generated by two distinct channels: nimodipine-sensitive (L-type) and -insensitive (non-L-type) channels (Rodriguez-Con-
treras and Yamoah 2001). Figure 2, A–D, shows examples of single-channel Sr\(^{2+}\) currents recorded from hair cells isolated from the frog saccule. Data in Fig. 2, A and B, represent Bay K 8644–modified currents. In contrast, the non-L-type channel currents were recorded with patch-pipettes containing 10 \mu M nimodipine (Fig. 2, C and D). We examined the permeation phenotype of the two-channel subtypes using different concentrations of patch-pipette Sr\(^{2+}\). For the traces shown, the amplitude histograms at different step potentials were generated to determine the mean single-channel amplitude as shown in Fig. 2E, and the resultant current-voltage relations yielded single-channel conductances (\gamma, in pS) of 17, and 8 for 70 and 5 mM, respectively for the L-type channel, whereas 18 and 13 pS were obtained for the same concentrations of Sr\(^{2+}\) for the non-L-type channel. The mean values for the single-channel conductances for Sr\(^{2+}\) followed in descending sequence from 70 to 5 mM for the L-type and non-L-type channels (Rodriguez-Contreras and Yamoah 2001). Respectively, they are 16.2 ± 1.9 and 18.7 ± 1.7 for 70 mM (n = 6) and 11.2 ± 1.8 and 13.3 ± 2.6 for 5 mM (n = 5).

To determine the sensitivity range of Ca\(^{2+}\) channels in hair cells for Sr\(^{2+}\), we examined the relation between the single-channel conductances versus Sr\(^{2+}\) concentrations. Using a Langmuir isotherm, a fit to the data accrued from recordings from the L-type channel (Fig. 3A) and the non-L-type channel (Fig. 3B) showed two important features of the channels: 1) the apparent dissociation constants of L- and non-L-type channels for Sr\(^{2+}\) ions were (in mM) 5.2 ± 1.9 and 1.9 ± 1.5 (n = 7), respectively; 2) meanwhile, the maximum conductances for the two channels were (in pS) 16.6 ± 1.4 and 19.2 ± 1.9 (n = 7) for the L- and non-L-type channels, respectively, and were reached at external Sr\(^{2+}\) concentrations above ~20 mM. Sim-

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**FIG. 4.** Family of single channel [dihydropyridine-sensitive (L-type)] traces recorded in 5 and 70 mM Sr\(^{2+}\). A: exemplary consecutive single channel traces were generated at different test potentials (indicated on top of each column of traces). The holding potential was −70 mV and the concentration of Sr\(^{2+}\) in the patch pipette was 5 mM. The ensemble averaged current traces shown below the single channel current traces were derived from ~150 consecutive sweeps. At 5 mM Sr\(^{2+}\), the ensuing ensemble averaged currents were sustained in nature. The scale bars (horizontal = 100 ms and vertical = 1 pA). B: similar consecutive sweeps of single channel current traces obtained when the patch pipette contained 70 mM Sr\(^{2+}\). The vertical scale bar = 2 pA; the horizontal bar = 100 ms. The holding potential was −70 mV and the step potentials are indicated. Note that the ensemble averaged currents derived from ~150 traces showed a slow decay.
ilar ranges of results have been reported for Ca\(^{2+}\) and Ba\(^{2+}\) currents (Rodriguez-Contreras et al. 2002). The ascending limbs of the conductance versus concentration curves for the two channel subtypes suggest that the sensitivity range of the channel for the permeant ion lies between 1 and 5 mM. Thus optimum assessment of Ca\(^{2+}\) channel functions should be evaluated within these narrow ranges of concentrations.

To ascertain whether the concentrations of Sr\(^{2+}\) ions have noticeable effects on the voltage dependence and kinetics of the channels, we examined the properties of the L-type channel at nonsaturating (5 mM) and saturating (70 mM) concentrations. To ensure that the recordings were made on single L-type channels, only patches containing single channel events that were blocked by nimodipine (5 \(\mu\)M) were analyzed. Additionally, kinetic analyses were performed on patches, which contained only one channel. The criteria consisted of quantitative determination followed by visual inspection of the data. The patches contained one channel, because there was no stacking of events. Furthermore, direct transition from fast to slow kinetics with similar current amplitude, and vice versa, were observed in the selected recordings, indicating that the gating modes were derived from a single channel. Figure 4 is a layout of cell-attached recordings of a family of consecutive single channel current traces, recorded using 5 (A) and 70 mM (B) Sr\(^{2+}\) as the charge carrier. With 5 mM Sr\(^{2+}\), the dwell times of the openings were frequent and long compared with recordings in 70 mM. Another visible difference was that, at low concentrations of the permeant ion, the openings were sustained, which was reflected in the persistent ensemble-averaged currents shown below the corresponding family of traces. In contrast, in 70 mM Sr\(^{2+}\), the channel had frequent openings predominantly only at the early phase of the step voltage, which is readily visible in the well-resolved decay of the ensemble-averaged currents. Notwithstanding the presence of 5 \(\mu\)M Bay K, an agonist of L-type channels, in the bath and pipette solutions, the probability of channel opening (\(P_o\)) was invariably <1 (Fig. 5). The rightward shift in the voltage dependence of activation as the pipette Sr\(^{2+}\) concentrations were raised from 5 to 70 mM is in keeping with surface charge screening effect of divalent cations (Rodriguez-Contreras and Yamoah 2003; Rodriguez-Contreras et al. 2002; Zhou and Jones 1995). The inference from the finding that the \(P_o\) was less than ~0.5 is that the channel has multiple nonconducting states. Indeed, by increasing the Sr\(^{2+}\) concentrations (70 mM), fluctuations of the open probability (\(P_o\)) plummeted in comparison to reduced concentrations (5 mM; Figs. 5 and 6, A and B), suggesting that the dwell times in the closed states may be current or ion concentration dependent. For graphic illustrations, we depict the voltage and time dependence of the normalized \(P_o\) of recordings performed at Sr\(^{2+}\) concentrations of 70 (Fig. 6A) and 5 mM (Fig. 6B). The gating of the channel was noticeably altered in several ways: 1) at saturating concentrations of Sr\(^{2+}\) (70 mM), nonconducting states are more favored than open states; 2) although channel openings are stochastic in nature, the \(P_o\) of the channel remains infinitesimally low at negative step potentials (~60 to ~40 mV), and even at step potentials greater than ~40 mV, the increase in \(P_o\) is relatively modest compared with recordings done at Sr\(^{2+}\) concentrations (5 mM) within the sensitivity range of the channel; 3) finally, at the steeper phase on the sensitivity curve of the channel, the \(P_o\) of the channel was high at negative voltages and remained so at more positive voltages, indicating that, at low concentrations of the permeant ion, the channel has increased propensity to exit from the nonconducting states. The shift in the activation of the channel using 5 and 70 mM Sr\(^{2+}\) is shown in the normalized \(P_o\) versus voltage plot (Fig. 6C). The midpoints of activation (in mV) were ~24.1 ± 1.2 and ~13.7 ± 1.5 (\(n = 7\)), and the slope factor of the curves were virtually unchanged (in mV) at 4.3 ± 0.7 and 4.4 ± 0.5 (\(n = 7\)) for 5 and 70 mM Sr\(^{2+}\), respectively. Moreover, the slope of the relation between the midpoint of activation and Sr\(^{2+}\) concentrations (0.20 VM\(^{-1}\); Fig. 6D) was similar to the value obtained for Ca\(^{2+}\) as the permeant ion (Rodriguez-Contreras and Yamoah 2003). Although the ~10 mV rightward shift in the activation curves as the external divalent cation concentration was increased from 5 to 70 mM was consistent with surface charge screening effects, the value fell short of the predicted shift assuming the absence of permeant ion binding interaction with surface charges (~25 mV) (Rodriguez-Contreras and Yamoah 2003; Zhou and Jones 1995). Thus Sr\(^{2+}\) binding to the surface charge may dictate the extent of the shift in the activation curve (Rodriguez-Contreras and Yamoah 2003).

If indeed the gating of the channel is altered by the permeant ion concentrations, and that at saturating levels the transition rate constants are altered, we would expect the waiting time to first opening or the first latency distribution to change in recordings performed at the two different spectrums of the sensitivity range of the channel. Figure 7 shows the normalized cumulative first latency distribution at 70 (A) and 5 mM Sr\(^{2+}\) (B) in the patch pipette solution. In accord with a voltage-dependent channel, the time constant of the first latency (\(\tau_{ls}\)) was faster at depolarized than at hyperpolarized voltage steps. However, in keeping with the notion that the transition between closed states may be ion concentration dependent, \(\tau_{ls}\) were starkly faster in recordings performed in pipette Sr\(^{2+}\) concentrations that lay within the most sensitive phase of the channel’s conductance–concentration relations (Fig. 3) than at saturating concentrations. Figure 7C compares the \(\tau_{ls}\) of Ba\(^{2+}\), Ca\(^{2+}\), and Sr\(^{2+}\) to assess the divalent cations that may promote multiple closed states or reluctance to transition from the closed states to first openings.
Another direct way to determine the effects of charge carrier concentration on the kinetics of Sr\(^{2+}\) currents is to obtain the open and closed time distributions from the single-channel records. Figure 8 shows open and closed time distributions at Sr\(^{2+}\) concentrations of 70 (Fig. 8, A and B) and 5 mM (Fig. 8, C and D). At 70 mM Sr\(^{2+}\), channel gating could be described by two open and two closed states, whereas at 5 mM Sr\(^{2+}\) (Fig. 8, C and D), an additional open state was included in the fit of open time distributions. As shown, patch depolarization increased the duration of the open states, whereas it had the opposite effect on the duration of the closed states. In addition, the time constant of longer duration in both closed and open time distributions was more frequent than the time constant of brief duration as judged from the relative areas of each component. We also show the relation between open and closed state duration and the membrane potential at 70 (Fig. 9, A and B) and 5 mM Sr\(^{2+}\) (Fig. 9, C and D). Comparison of open and closed times suggests that, at 5 mM, when the channel is at its...
sensitivity range, not only does the dwell time of the open state increase, but also the dwell time of the closed state plummets. It is the opposite case for recordings at saturating concentrations of Sr$^{2+}$/H$^{+}$ (70 mM).

**DISCUSSION**

Here, we report on the first extensive single-channel analysis of Sr$^{2+}$/H$^{+}$ currents in native hair cells. These recordings showed elementary properties that are usually impossible to identify from reports that use saturating concentrations of divalent cations to study Ca$^{2+}$/H$^{+}$ channels. By determining the sensitivity ranges of the channels and by using saturating and nonsaturating concentrations of Sr$^{2+}$/H$^{+}$, nuances of the permeation and gating characteristics of the channels that are prone to both permeant ion concentration and surface charge screening effects, are shown. First, we showed that macroscopic Sr$^{2+}$/H$^{+}$ currents essentially mirror the profile of the current of the physiological charge carrier Ca$^{2+}$/H$^{+}$ in magnitude and activation. The inactivation of whole cell Sr$^{2+}$/H$^{+}$ currents is much more similar to Ca$^{2+}$, although subtle differences are apparent, which is in keeping with reports that Sr$^{2+}$ can substitute for Ca$^{2+}$ in several biological processes (Abdul-Ghani et al. 1996; Xu-Friedman and Regehr 1999). Second, these findings show that, for Ca$^{2+}$/H$^{+}$ channel subtypes, such as the Ca$_{1.3}$ channels in hair cells that are subjected to Ca$^{2+}$-dependent inactivation, mediated by increase in intracellular Ca$^{2+}$ within a few angstroms of the channel via calmodulin binding (Peterson et al. 2000; Rodriguez-Contreras and Yamoah 2003; Shen et al. 2006; Yang et al. 2006), outcomes of using high concentrations of the permeant ion should be interpreted with the following caveats. Specifically, analyses of the open probability, time constants of openings and closures, and the kinetics of first latency of openings can be influenced considerably by the concentration of the permeant ion. Additionally, these findings should be substantiated with the underpinnings of the key features of the handling of the divalent cation used in the study (Carter et al. 2002; Xu-Friedman and Regehr 1999). Third, increased $P_o$, number of open states, and reduced dwell times of closed states of the channels in the physiologically important 1- to 5-mM range promote the increase in whole cell current amplitude and duration. Thus in the functional context of the channels as biological Ca$^{2+}$/H$^{+}$ transporters, the cellular physiological implications of these findings could be vast.

By virtue of its similar charge and size, Sr$^{2+}$ substitutes well for Ca$^{2+}$ in mediating synaptic transmission at the squid giant synapse (Augustine and Eckert 1984), neuromuscular junction (Bain and Quastel 1992; Dodge et al. 1969), and synapses in
the brain (Tokunaga et al. 2004; Xu-Friedman and Regehr 1999), albeit at different kinetics. Indeed, Sr\textsuperscript{2+} can maintain mechanoelectrical transduction (Ohmori 1985) and activate Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels in hair cells (Sugihara 1998) and in other systems (Oberhauser et al. 1988). However, because intracellular Ca\textsuperscript{2+} buffers are less efficient in chelating Sr\textsuperscript{2+} than Ca\textsuperscript{2+} (Yawo 1999), replacing pipette Ca\textsuperscript{2+} with Sr\textsuperscript{2+} was predicted to reduce the influence of Ca\textsuperscript{2+}-dependent modifications and intracellular Ca\textsuperscript{2+} buffers. Thus we surmised that Sr\textsuperscript{2+} entry through Ca\textsuperscript{2+} channels would be more suitable to examine the elementary properties of voltage-gated Ca\textsuperscript{2+} channels in hair cells than Ca\textsuperscript{2+} flux itself, which is confounded by Ca\textsuperscript{2+}-dependent buffering and signaling.

Previous analyses of the concentration dependence of unitary current and conductance to determine the $K_D$ of Ca\textsuperscript{2+} channels have shown that the concentration versus conductance curves provide a more reliable measure since they are independent of surface charge screening effects and voltage errors introduced by liquid junction potentials (Church and Stanley 1996; Rodriguez-Contreras and Yamoah 2003; Rodriguez-Contreras et al. 2002). From the calculated maximum conductances and the $K_{D}$s of the two Ca\textsuperscript{2+} channel subtypes, at the equivalent physiological Ca\textsuperscript{2+} concentration of ~2 mM, the conductance (in pS) of Sr\textsuperscript{2+} for the L- and non-L-type channels were 1.5 and 4.0, respectively. These conductance values dovetail well with extrapolated data from concentration dependence curves of Ca\textsuperscript{2+}, which were ~1.0 and 2.7 pS for the L- and non-L-type channels (Rodriguez-Contreras et al. 2002). Indeed, direct measurement of the physiological Ca\textsuperscript{2+} conductance of the L-type channel yielded ~1.2 pS (Rodriguez-Contreras et al. 2002), which is within the range of these results. Thus at a membrane potential of hair cells (e.g., ~65 mV), and a reversal potential for Sr\textsuperscript{2+}/Ca\textsuperscript{2+} of ~100 mV, the limits of the flux rate of Ca\textsuperscript{2+} ions ranges from 500 to 1,400 ions/ms and that of Sr\textsuperscript{2+} ions ranges from 800 to 2,000 ion/ms. For clustered channels in hair cells, these unitary Ca\textsuperscript{2+}/Sr\textsuperscript{2+} domains are expected to increase by ~100-fold (Roberts et al. 1990; Rodriguez-Contreras and Yamoah 2001). Another essential feature of the concentration dependence curves is the difference in the steepness at 1–5 mM (Sr\textsuperscript{2+}: 0.36 pS/mM; Ca\textsuperscript{2+}: 0.34 pS/mM) and 10–70 mM (Sr\textsuperscript{2+}: 0.08 pS/mM; Ca\textsuperscript{2+}: 0.04 pS/mM), suggesting that the channels are ~8-fold and 4-fold more sensitive at physiological concentration ranges of Ca\textsuperscript{2+} and Sr\textsuperscript{2+}, respectively. By retaining high selectivity and sensitivity, an optimal throughput of divalent cations is ensured at physiological concentration ranges.

A question that arises from this study is how can one evaluate the respective roles of subsaturating and saturating concentrations of permeant ions on 1) permeation, as it relates to means by which the permeating ions interact with binding sites within the pore of the channel (Kuo and Bean 1993); 2) allosteric effects of binding of divalent and/or monovalent ions to sites of the channel that mediate changes in protein conformational changes that produce alterations in gating mechanism (Zamponi and Snutch 1996); and 3) last, electrostatic actions of different concentrations of divalent ions on the membrane electric field (Hille 2001; Zhou and Jones 1995). Our Sr\textsuperscript{2+} current amplitudes and conductances ($\gamma$) at saturating concentrations are consistent with those reported previously for Ca\textsuperscript{2+}, 1.2, 1.3, 2.2, and 2.3 channels, which follow the sequence ($\gamma_{Ba} > \gamma_{Sr} > \gamma_{Ca}$) (Fox et al. 1987; Rodriguez-Contreras and Yamoah 2003). Moreover, comparison of the conductance obtained at subsaturating concentrations of divalent ions was within the range of prior observations (Rodriguez-Contreras et al. 2002).
and Yamoah 2003). In particular, the overall sequence in unitary conductances at subsaturating concentrations (5 mM) was distinct from data obtained at saturating concentrations (70 mM) for both channels (at 5 mM L-type: $\gamma_{Ba} = \gamma_{Sr} > \gamma_{Ca}$, non-L-type: $\gamma_{Sr} > \gamma_{Ba} = \gamma_{Ca}$). Of note, the results cannot be accounted for by the shifts in the activation curves from high to low concentrations of the permeant ions.

Although it is difficult to disentangle the effects of surface charge screening from allosteric effects, it has been shown that the binding of Ca$^{2+}$ to Ca$_{2.1}$ channels produces functional allosteric mechanisms that alter channel gating (Zamponi and Snutch 1996). In this study, we showed that changing the concentration of Sr$^{2+}$ from saturating to subsaturating levels profoundly alters the gating of the Ca$_{1.3}$ channels. In particular, at subsaturating levels, the Ca$_{2+}$ channel exhibits increased long openings and a reduced closed time. Because both charge screening and selective binding of Sr$^{2+}$ (Hille 2001) to the channel cannot account for these findings, we suggest that an allosteric interaction of Sr$^{2+}$ with the L-type channel in hair cells may mediate a state-dependent alterations of a closed state (Gilly and Armstrong 1982a,b), which is in keeping with the ensuing alterations of the first latency distributions (Fig. 7). Specifically, the time constant of the first latency distribution was shorter at subsaturating concentrations of Sr$^{2+}$ than at saturating levels.

The physiological interest of this study lies in the increased realization that Ca$^{2+}$ channels in hair cells may exhibit strong Ca$^{2+}$-dependent inactivation (Rodriguez-Contreras and Yamoah 2003; Shen et al. 2006; Song et al. 2003; Yang et al. 2006) that may vary during different stages of development and regeneration (Levic et al. 2007; Yang et al. 2006) against the backdrop of previous reports that hair cells use an efficient Ca$^{2+}$-buffering mechanism (Roberts 1993) that may mask the genuine phenotype of Ca$^{2+}$ currents. It is also clear that the Ca$^{2+}$ channels are extremely sensitive at subsaturating concentrations of the permeant divalent ions, and we can infer that Ca$^{2+}$-dependent inactivation might actually have dramatic effects on Ca$^{2+}$ currents at physiological Ca$^{2+}$ concentrations at the resting potential because the channels have a sizable $P_o$ at baseline. Analysis of the Ca$^{2+}$ handling at the subnanometer Ca$^{2+}$ domains of the Ca$^{2+}$-channel in hair cells will show further details of the basic properties of the channel as a physiological Ca$^{2+}$ transporter.

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