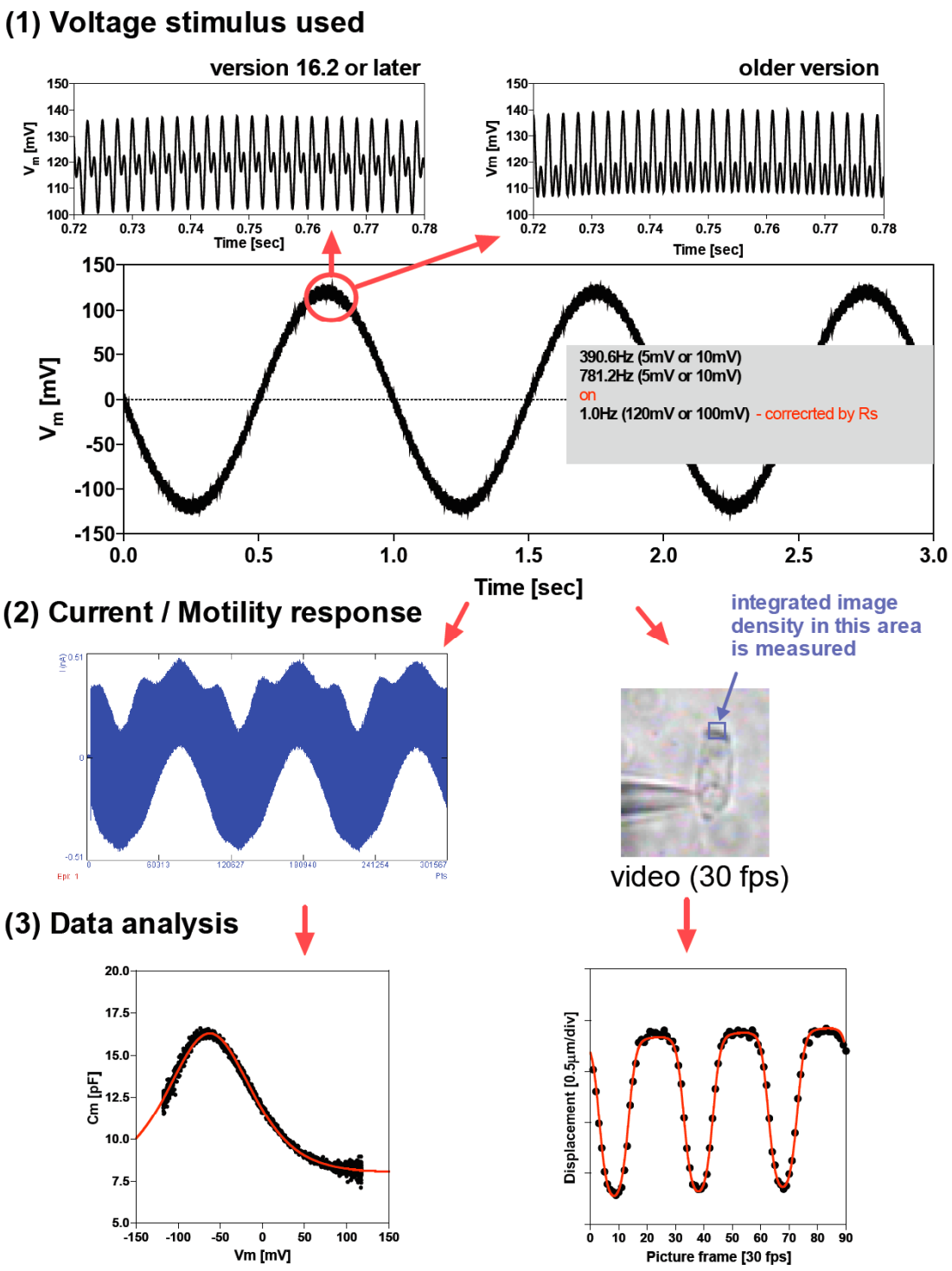

Figure S1. Simultaneous measurement of NLC and OHC electromotility. NLC and motility were simultaneously measured in the whole-cell configuration shown above at room temperature with holding potential at 0 mV. Sinusoidal voltage stimulus (1-Hz, 120mV amplitude or 2-Hz, 100mV) superimposed with two higher frequency stimuli (390.6 and 781.2 Hz, 5mV or 10mV amplitude) was used. The actual voltage applied to a cell was determined after correction for series resistance. OHC electromotility was captured by the 30-fps digital camera. The spatial resolution of the motility analysis is mainly determined by the objective lens and the pixel size of the camera. The spatial resolution of our system was 0.67 µm/pixel, which is not high enough for analyzing the typical mouse OHC displacement of 1~2

μm. However, by using the 8-bit gradient information of 5x5 pixels positioned at the apical region of an OHC, we were able to follow OHC electromotility with 45 nm resolution. This resolution was determined using the same voltage protocol on OHCs derived from prestin-ko mice. The standard deviation of the position of the apical region of the cell during the voltage stimulation was used for defining the resolution. The displacement of an OHC was tracked by monitoring the image density of the 5x5 pixels window, and the image density was analyzed using ImageJ program. For determining actual OHC displacement from image density, the 5x5 pixel window was manually moved pixel by pixel along the axis of the OHC electromotility on one of the sequential images of a motility movie. Calibration curve was generated by using the known pixel size (0.67 μm/pixel). It is important to generate a new calibration curve for each analysis, because the apparent darkness of the cuticular plate of OHCs varies from one cell to another. Both NLC and motility data were analyzed by the 2-state Boltzmann model-based equations (Eq.3 and Eq.6) and the 3-state Boltzmann model-based equations (Eq.4 and Eq.7). For curve-fitting motility data, the V_m term in Eq.6 and Eq.7 was simply replaced with the sinusoidal voltage stimulus (1Hz, 120 mV amplitude) as follows,

$$V_m = V_0 \cos[\frac{2\pi x}{N}]$$

where V_0 is the amplitude of the voltage stimulus (120mV), x is the frame number of the sequential images, and N is the number of pictures the camera takes per a sinusoidal stimulation cycle (30 for 1-Hz, 15 for 2-Hz). Conventionally, OHC displacements are induced by step voltage stimuli, and the displacement is determined for each voltage step, and replotted against the voltage for curve-fitting analysis. Our method does not require replotting. The fitting parameters and the standard errors of those parameters are immediately obtained by a single curve-fitting analysis.

Current data were collected by jClamp version 18 (SciSoft Company, New Haven, CT) for the fast Fourier transform-based admittance analysis for determining NLC (Santos-Sacchi et al., 1998). In the dual sinusoidal method for determining NLC, 10mV is typically used for the amplitudes of the two superimposed small sinusoidal stimuli. The fundamental premise for precisely determining membrane capacitance with this method is that the small voltage amplitude (10 mV) does not induce nonlinear response. This is, of course, not the case for prestin's NLC, and hence the name. As an attempt to further minimize the nonlinearity of the response for better comparison of NLC vs. motility, we used 5 mV amplitude for the Boltzmann-parameter comparisons between NLC and motility (Fig. 4). Since 10 mV amplitude gave very similar results, we also used 10 mV for some measurements (Figs. 1, 2, and 5). It should be noted that there is $\pi/2$ phase shift between the two small sinusoidal voltage stimuli in older versions of jClamp, which makes the overall voltage stimulus asymmetric. In our preliminary NLC-motility measurements with versions 14 and 15 with 10 mV amplitude for the two small sinusoidal voltage stimuli, we found significant discrepancy in V_{pk} derived from motility and NLC. V_{pk} values determined by motility measurements were typically 10~20 mV higher (depolarized) than those determined by NLC measurements, while discrepancy in α was not significant. This is understood by he asymmetry generated by the $\pi/2$ phase difference, which would induce non-negligible nonlinear response. Motility measurement is also affected by this asymmetry. Small OHC displacements induced by the superimposed two small sinusoidal stimuli are averaged because motility is recorded at 33 msec/frame. In the absence of asymmetry in the voltage stimulus, the position of the apical boundary of an OHC determined by an averaged image density will be the same as that determined without the superimposed small voltage stimuli. With an asymmetry, however, a higher image density will be obtained at a shifted position irrespective of the nonlinearity of prestin's voltage response.

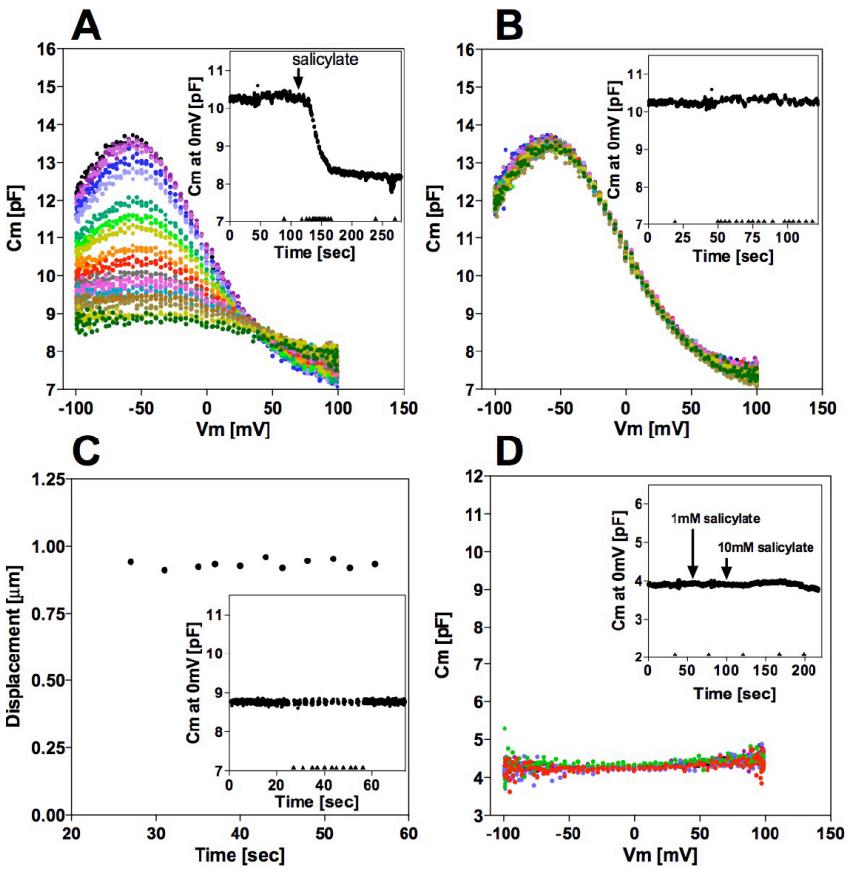

Figure S2. Effects of salicylate and repetitive stimulation on OHCs. All insets show NLC recordings or motility recordings, which were performed in the whole cell configuration at time points indicated by the solid triangles, while the membrane capacitance at 0 mV was constantly monitored during the measurements. The time-abscissa indicates the time after establishing the whole cell configuration. **(A) Salicylate inhibits prestin-dependent charge movement.** NLC was measured on an isolated OHC repetitively before and after applying 1.5 mM salicylate in the bath solution. Different colors represent different NLC recordings at different time points. **(B) Repetitive voltage stimulation does not affect NLC.** Repetitive NLC measurements were performed as in (**A**) except for application of salicylate. Different colors represent different NLC recordings at different time points shown in the inset. The C_m values at +100mV showed slight fluctuation among recordings, however, a tendency of a constant change (decrease or increase) was not found during the 18 repetitive measurements. **(C) Repetitive voltage stimulation does not affect OHC electromotility.** Repetitive electromotility measurements were performed at different time points shown in the inset in the absence of salicylate. **(D) Salicylate does not affect the basal linear membrane capacitance in the absence of prestin.** NLC measurements were performed on an OHC derived from prestin-ko mouse under various concentration of salicylate. Different colors represent different NLC recordings at different time points shown in the inset.

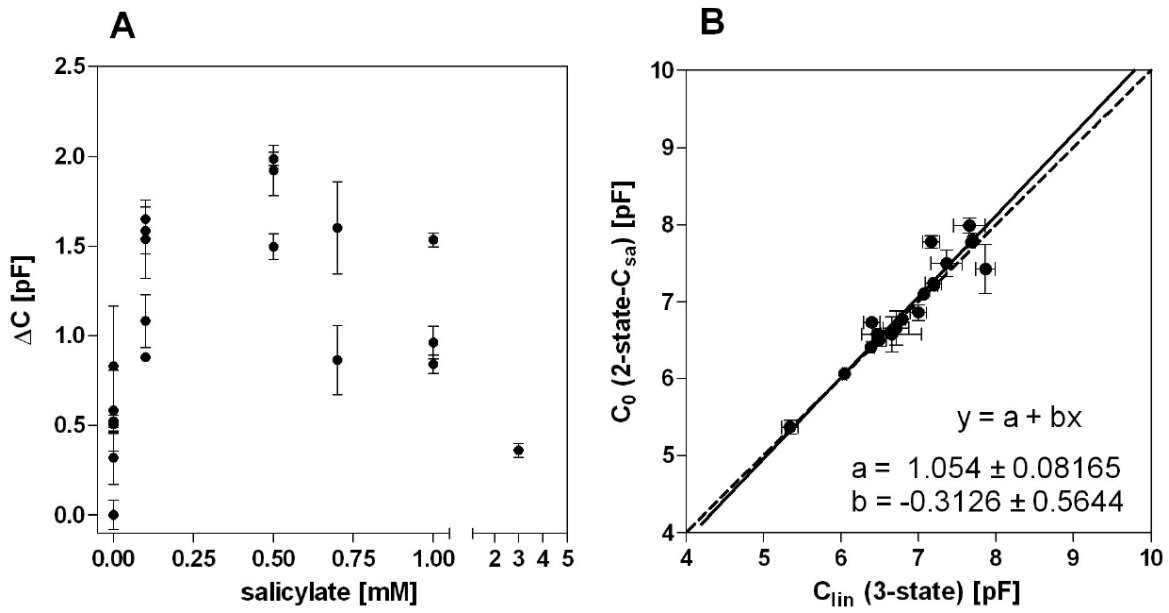

Figure S3. Salicylate-dependent ΔC and comparison of the linear capacitances determined by the 3-state and the 2-state-C_{sa} models. NLC was measured on individual OHCs in the whole-cell configuration in the presence of various concentrations of salicylate in a steady-state condition. For each measurement, salicylate concentrations in the intracellular solution and the extracellular solution were the same. The error bars indicate the standard error of fitting. **(A)** Salicylate dependence of ΔC determined by the 2-state-C_{sa} model. **(B)** Comparison of C_{lin} and C_0. The basal linear capacitances determined by the 3-state (C_{lin}) and the 2-state-C_{sa} (C_0) models were compared. The diagonal broken lines indicate exact match (y = x). Deming linear regression analyses (solid lines) followed by *t*-tests were performed for testing identity (see Materials and Methods). The fitting parameters determined by the Deming linear regression analysis are shown in the figure along with the standard errors. The p-values for the y-intercept and the slope were 0.59 and 0.52, suggesting that the two different models estimate very similar linear capacitances.

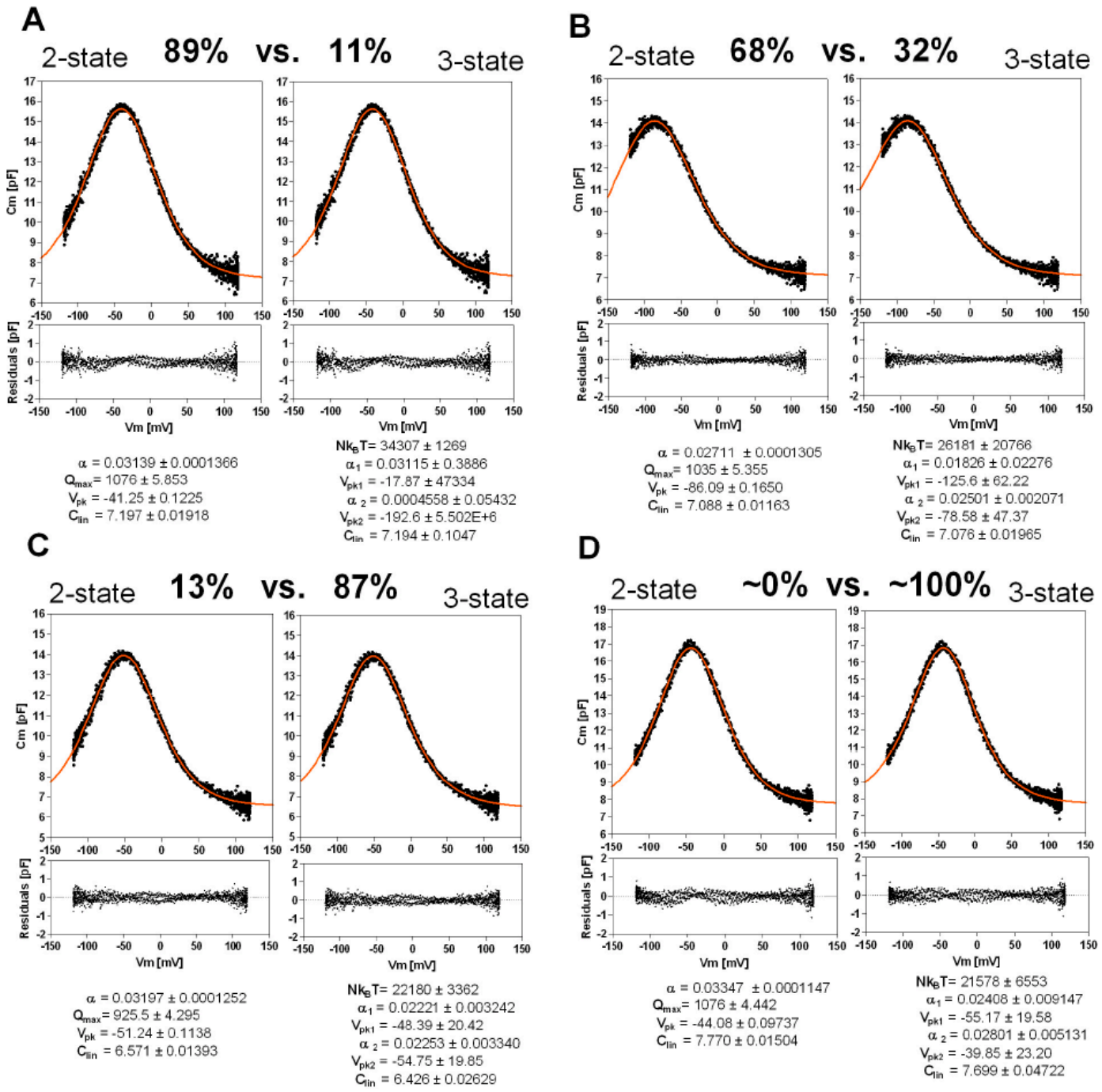

Figure S4. Comparison of the 2-state and the 3-state Boltzmann fittings on NLC data recorded in the absence of salicylate. Four examples of NLC data recorded in the absence of salicylate. Equations used for the curve fittings are Eq.3 and Eq.4 for the 2-state and 3-state Boltzmann fittings, respectively. Akaike's information criterion (AICc) method was used for comparing the goodness of fit. Akaike's weights, which represent the likelihood of one model to be correct over an alternative model, are shown on the top of each comparison, (**A**) 89% vs. 11%, (**B**) 68% vs. 32%, (**C**), 13% vs. 87%, (**D**) ~0% vs. ~100%. Note that Akaike's weight is a relative index, which is only used for a given comparison. Note that the 3-state model does not always improve fittings, and usually generates large standard errors for the fitting parameters.

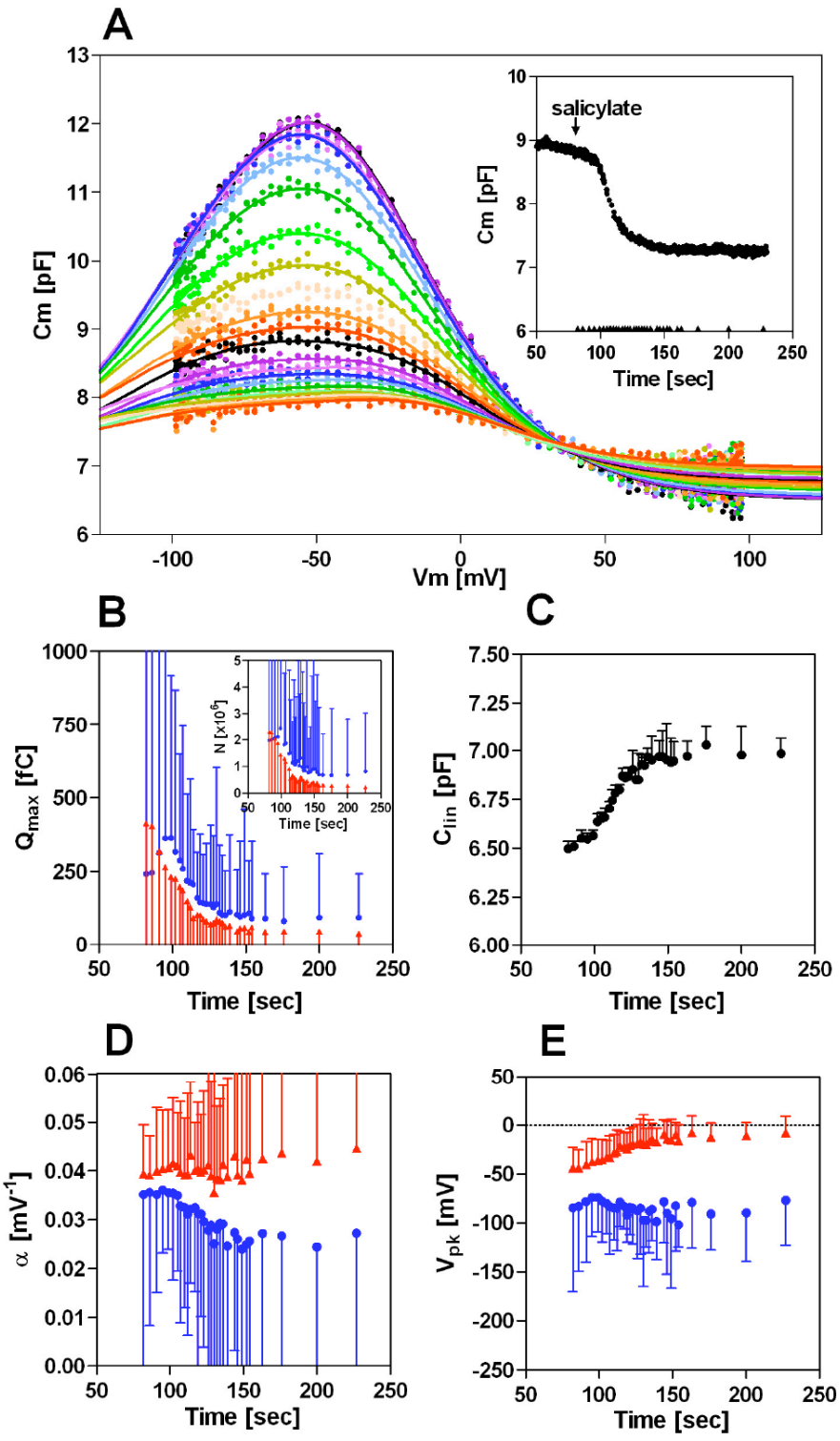

Figure S5. Analysis of Salicylate-dependent NLC data using a 2x2-state model. (A) NLC was repetitively measured from an isolated OHC before and after applying 1.5 mM salicylate in the bath solution. Different colors represent different NLC recordings at different time points shown in the inset. The solid lines are fitting curves by the 2x2-state model described as

$$C_{m2x2-state} = \frac{\alpha_1 Q_{\max 1} K_1}{(1+K_1)^2} + \frac{\alpha_2 Q_{\max 2} K_2}{(1+K_2)^2} + C_{lin}$$

where K_1 and K_2 are equilibrium constants that are expressed as $\exp\{\alpha_1(V_m-V_{pk1})\}$ and $\exp\{\alpha_2(V_m-V_{pk2})\}$, respectively. Q_{maxi} can be expressed as $\alpha_t N_i k_B T$ for the consistency to the equations shown in the main manuscript. Q_{maxi} is proportional to the population of each prestin component (chloride-bound or salicylate-bound). The seven NLC parameters, Q_{max1}/Q_{max2}, (or N_1/N_2), C_{lin}, α_1/α_2, and V_{pk1}/V_{pk2} were determined for each NLC curve, and were plotted against the recording time (**B**~**E**). The blue symbols represent Q_{max1}, α_1, or V_{pk1} while the red symbols represent Q_{max2}, α_2, or V_{pk2}. The bars indicate the standard errors of fitting. The magnitudes of the standard errors are larger than those found in the 3-state model because of the increased number of the free fitting parameters (six for the 3-state model vs. seven for the 2x2-state model). As seen in panel B, both components decline monotonically.

Correction of a electromechanical coupling efficiency, m

N molecules of prestin motors are two-dimensionally distributed in the lateral membrane of an OHC, whose area is calculated as LDπ, where L is the length, and D is the diameter of the OHC. For simplification we neglect the lack of prestin at the cell's nuclear pole and in its cuticular plate region (1,2). If we assumed uniform distribution of prestin throughout the lateral membrane, and the linear density of prestin in the lateral membrane were defined as *p*, N is expressed as

$$N = pL \times pD\pi$$

Therefore, *p* is computed as

$$p = \sqrt{\frac{N}{LD\pi}}$$

The axial OHC displacement, *d*, is determined by

$$d = mq_u N_{axial}$$

where q_u (= ze) is the unitary charge movement, and N_{axial} is the number of molecules that align in the axial direction of the cell, which can be described as

$$N_{axial} = Lp = L\sqrt{\frac{N}{LD\pi}}$$

Since Eq.9 takes N, but not N_{axial}, into account for determining *d*, m values directly obtained from Eq.9 (m_{calc}) has to be corrected by the ratio of N to N_{axial}. Therefore, the true m value will be calculated as

$$m = m_{calc} \times \frac{N}{N_{axial}} = m_{calc} \times \frac{N}{L\sqrt{\frac{N}{LD\pi}}}$$

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2. Huang, G., and Santos-Sacchi, J. (1993) *Biophys J* **65**, 2228-2236