Structural rearrangements of the motor protein prestin revealed by fluorescence resonance energy transfer

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The mammalian hearing organ is unique in its exquisite sensitivity and frequency resolving capacity. These features, which have enabled sophisticated speech and music in humans, are imparted in large part by the outer hair cells (OHCs) in the cochlea that is essential for hearing. This unique motor protein transduces a change in membrane potential into a considerable mechanical force, which leads to a cell length change in the OHC. The nonlinear capacitance in cells expressing prestin is recognized to reflect the voltage-dependent conformational change of prestin, of which its precise nature remains unknown. In the present work, we aimed to detect the conformational changes of prestin by a fluorescence resonance energy transfer (FRET)-based technique. We heterologously expressed prestin labeled with fluorophores at the COOH- or NH2-termini in human embryonic kidney-293T cells, and monitored FRET changes on depolarization-inducing high KCl application. We detected a significant decrease in intersubunit FRET both between the COOH-termini and between the COOH- and NH2-termini. A similar FRET decrease was observed when membrane potential was directly and precisely controlled by simultaneous patch clamp. Changes in FRET were suppressed by either of two treatments known as nonlinear capacitance (NLC) (28). NLC is generally accepted as the electrophysiological signature of motor action and is commonly used to assess prestin function in heterologous expression systems. It is also known that intracellular chloride is essential for proper prestin function and may act as a voltage sensor or as an allosteric modulator (17). However, the precise nature of the voltage-dependent conformational changes that confer motility and voltage-sensing ability are unknown. We used fluorescence resonance energy transfer (FRET) in combination with total internal reflection fluorescence (TIRF) microscopy to investigate the molecular rearrangements of prestin upon change in membrane potential. FRET describes a distance-dependent energy transfer mechanism of excited state energy from a donor fluorophore to an acceptor fluorophore. The efficiency of energy transfer is extremely sensitive to the separation between fluorophores, making FRET a sensitive and powerful technique for studying the structure and conformational changes of proteins. FRET techniques have been successfully applied to the study of membrane protein structure, which is difficult to interrogate using traditional structural techniques such as X-ray crystallography and nuclear magnetic resonance. In addition, FRET can be used to obtain dynamic information about the structural rearrangements that proteins undergo upon external stimuli, like a ligand-binding event or a change in membrane potential (2, 8, 18, 22).

The present work discusses changes in FRET that were detected between individual prestin subunits labeled with fluorophores at the COOH- and NH2-termini. Our results are consistent with significant movements in the COOH-terminal domain of prestin upon change in membrane potential, provid-
ing the first dynamic information on the molecular rearrangement of prestin.

MATERIALS AND METHODS

Constructions and expression system. Cerulean fluorescent protein (CFP) (19) and Venus yellow fluorescent protein (YFP) (14) were attached to the full-length COOH-terminus of prestin, whereas the fluorescein arsenical hairpin (FIAshH)-targeting sequence CCReCC was appended to the full-length NH2-terminus (1). All constructs were made using PCR-based methods and expressed in the pCXN2 expression vector (16). Human embryonic kidney cells (HEK-293T) were cultured on glass-bottomed dishes (Matsumata Glass, Osaka, Japan) and transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA). Experiments were carried out 24–48 h after transfection.

FIAshH. Labeling was carried out basically as described by the supplier’s manual. Between 24 and 48 h posttransfection, HEK-293T cells were washed twice with phenol red-free Hank’s balanced salt solution containing 1 g/l glucose (HBSS) and incubated away from light at room temperature for 1 h with 1 μM FIAshH reagent (TC-FIAshH II, Invitrogen) suspended in HBSS containing 12.5 μM EDT. Cells were again rinsed twice with HBSS and incubated in HBSS containing 75 μM EDT for 15 min. Finally, cells were rinsed twice with HBSS and allowed to settle for at least 30 min before optical recording.

TIRF image acquisition and FRET analysis. Fluorescence from single cells expressing both CFP- and YFP-fused or CFP- and FIAshH-fused prestin was imaged and measured using a TIRF microscope equipped with an oil immersion objective (60×, 1.45 numerical aperture) and argon (515 nm) and helium-cadmium (424 nm) lasers (Olympus, Tokyo, Japan). Cerulean was excited by the 442-nm laser lines, and the emission signals from Cerulean and Venus or FIAshH via FRET were divided by DMS05 dichroic mirror (Chroma Technology, Rockingham, VT) mounted on image splitter (SIP-FRET, Olympus, Tokyo, Japan) and then passed through S470/30 and S535/30 (Chroma Technology, Rockingham, VT) filters, respectively. The divided images were amplified by an image intensifier unit (C8600, Hamamatsu Photonics, Shizuoka, Japan) and then simultaneously recorded using a cooled CCD camera (CoolSNAP, Roper Scientific, Hamamatsu Photonics, Shizuoka, Japan) and argon (515 nm) and helium-cadmium (424 nm) lasers equipped with an oil immersion objective (60×, 1.45 numerical aperture) and argon (515 nm) and helium-cadmium (424 nm) lasers (Olympus, Tokyo, Japan). The exposure time was 100 ms, and images were collected every 3 s. Background fluorescence, obtained from nontransfected cells, was subtracted from all images. Cells were superimposed continuously with bath solution by gravity at a rate of about 3 ml/min, and each concentration of glutamate was applied by changing the superfusion solution. Intermolecular FRET efficiency at each time point was estimated as the following:

\[
C_m(i) = (R/R_{exp})^2 (Q/V_c)
\]

where

\[
R_t = (R_t - \gamma) + \gamma V_c
\]

\[
R_{exp} = R_t - R_h
\]

\[
C_m(i) = C_{lin} + (Q_{max} - \alpha) [\exp(-\alpha(V_m - V_c)) - 1]
\]

where \(C_{lin}\) is linear capacitance, \(Q_{max}\) is maximum nonlinear charge transfer, \(V_c\) is voltage at half-maximal nonlinear capacitance, and \(\alpha\) is a slope factor.

Simultaneous recording by patch clamp and TIRF-FRET. Cells confirmed to show a decrease in FRET upon high KCl application were subsequently recorded on by whole cell patch clamp. First, the change of the membrane potential under the current clamp (CC) condition was monitored upon high KCl application, and then membrane current under voltage clamp (VC) was monitored. In both cases of CC and VC recordings, FRET changes were monitored simultaneously under TIRF microscopy. The details of the patch clamp and FRET recording conditions are the same as above, with the exception that a 140 mM KCl-based patch pipette solution (140 KCl, 3 MgCl2, 5 HEPES, 10 EGTA, 0.1 CaCl2, and 2.5 Na2ATP and adjusted to pH 7.3 with KOH) was used.

Analysis and statistics. The magnitude of change of FRET values was calculated by determining ΔF/F, where \(F\) was the baseline nF/FRET from the first 30 acquisitions, and ΔF/F was the average nF/FRET during high K+ solution application. All data were expressed as means ± SE. Statistical significance was evaluated by unpaired Student’s t-test or Dunnett’s t-test; values of \(P < 0.05\) were considered statistically significant.

RESULTS

Fluorescent labeling of prestin. FRET studies require a macromolecule be labeled with two kinds of fluorescent labels: a donor and an acceptor. In this case, CFP (19) was chosen as the donor fluorophore and YFP (14) was the acceptor. Initially, the fluorescent proteins were appended to both the COOH- and NH2-termini of prestin. Whereas the presence of the fluorescent protein at the COOH-terminus did not disrupt membrane expression or electrophysiological function (Fig. 1, A and B), the incorporation of fluorescent proteins at the NH2-terminus of prestin led to improper membrane targeting and loss of NLC (Fig. 1, C and D). Thus the less disruptive strategy of posttranslational labeling with a small molecule fluorophore (FIAshH) (1) was employed at the NH2-termini. As prestin is thought to function as an oligomer, both intersubunit (between COOH- and COOH-termini or NH2- and COOH-termini of different prestin molecules) and intrasubunit (between NH2- and COOH-termini of the same prestin molecule) interactions were assessed.
and COOH-termini of the same molecule) conformational changes were studied.

In the case of intersubunit FRET, equal amounts cDNA constructs of donor and acceptor fluorophore-labeled prestin were cotransfected into HEK-293T cells. Because the exact number of subunits that comprises a functional prestin oligomer is still under debate (7, 9, 13, 25), the issue of subunit stoichiometry could not be definitively addressed. However, our results clearly support the growing consensus that prestin operates as a functional multimer. Intersubunit FRET was observed between the COOH-termini of prestin, as determined by the FRET-to-donor fluorescence ratio (nF/\(I_{\text{CFP}}\)) = 0.30, n = 16) and donor dequenching (E = 0.16 ± 0.02, n = 14). In a donor dequenching (acceptor photobleaching) experiment, energy transfer efficiency is determined by the change in donor emission before and after selectively photobleaching the acceptor molecule. FRET was likewise observed between the COOH- and NH2-termini of different prestin subunits, although this could only be measured by nF/\(I_{\text{CFP}}\) (nF/\(I_{\text{CFP}}\) = 2.9 ± 0.3, n = 5), prestin labeled with both donor and acceptor fluorophores was cotransfected with wild-type, nonfluorescent prestin in a 1:10 ratio to minimize intersubunit FRET to an ignorable level.

Controlling membrane potential using extracellular K\(^+\) ion concentration. Prestin is accepted to undergo conformational rearrangements in response to changes in membrane potential. In most of the experiments in the present study, we chose to control membrane potential by varying extracellular K\(^+\) concentration. This approach leaves the mechanical properties of the membrane of the transfected cell unperturbed. The resting membrane potential of a HEK cell ranged from −40 to −80 mV depending on the leak conductance level. Application of a 140 mM KCl solution resulted in a membrane potential of about 0 mV, thus depolarizing the membrane. This change in membrane potential is within the range where NLC has been demonstrated for heterologously expressed prestin (Fig. 1A) and thus should be effective in inducing a conformational change. Furthermore, measurement of NLC at 140 mM KCl confirms that the NLC of prestin is retained under high extracellular K\(^+\) concentration conditions (data not shown).

Decrease in FRET is observed in response to high KCl application. Because changes in FRET, as opposed to absolute FRET values, were of primary interest, FRET efficiency was estimated as FRET-to-donor fluorescence ratio (nF/\(I_{\text{CFP}}\)). These values were then used to obtain the magnitude of the FRET change (∆F/F) that occurred upon application of membrane-depolarizing concentrations of KCl. Where appropriate, FRET efficiency was measured by donor dequenching techniques to gain a more accurate measurement of absolute FRET efficiency.

For the intersubunit combinations of prestin labeled at the COOH-termini with YFP (Pres-YFP) and CFP (Pres-CFP), a decrease in FRET efficiency was observed upon application of 140 mM KCl solution (Pres-YFP:Pres-CFP, ∆F/F = 0.091 ± 0.003, n = 16). FRET values returned to their initial levels upon reapplication of 4 mM KCl solution (Fig. 2A).
use of this combination, it was also possible to obtain a more rigorous measure of the magnitude of FRET change by doing donor dequenching experiments in the presence of either 4 mM KCl (normal) or 140 mM KCl (high). In the presence of a high K⁺ concentration, the fractional increase in the donor intensity upon dequenching (E) was 0.003 ± 0.03 (n = 14), approximately 50 times lower than the value obtained in the presence of 4 mM KCl (0.16 ± 0.02, n = 11), consistent with a significant conformational change between donor and acceptor fluorophores (Fig. 2C).

To confirm that the changes in FRET were due to prestin and not, for instance, global changes in cell morphology, FRET changes of the metabotropic glutamate receptor (mGluR) were monitored upon high K⁺ concentration application. For HEK cells transfected with a mixture of mGluR1 tagged with CFP or YFP, the FRET intensity increased upon application of activating concentrations of glutamate and decreased upon washout (Fig. 2B inset) (23), but no change was observed upon change in extracellular K⁺ concentration (Fig. 2B). In addition, visual monitoring of cells during the application of high K⁺ solution gave no detectable indication of gross morphological changes.

In the case of the FlAsH-labeled proteins, it was necessary to ensure that the FRET changes were not an artifact of nonspecific background labeling of the FlAsH molecule. In HEK cells transfected with both FlAsH-prestin and Prestin-CFP, a decrease in FRET was observed upon membrane depolarization (Fig. 3A). The value (∆F/ F = 0.11 ± 0.02, n = 8) was significantly higher than that observed in Prestin-CFP-transfected cells labeled with FlAsH in the absence of a FlAsH-labeling motif (CCRECC) (∆F/ F = 0.056 ± 0.01, n = 15, P < 0.05, Fig. 3C). The result demonstrates a significant FRET change between intersubunit NH₂- and COOH-termini upon membrane depolarization. Although a decrease in FRET was similarly observed in the case of FlAsH-Prestin-CFP, a reporter of intrasubunit FRET change, this decrease was not shown to have a statistically significant difference from that of negative control group (∆F/ F = 0.089 ± 0.02, P = 0.19, Fig. 3, B and C). When unlabeled CCRECC-prestin was coexpressed with Prestin-CFP, no FRET was observed, as expected from the absence of FRET acceptor (data not shown).

**FRET changes are related to NLC.** To determine whether the conformational changes were related to NLC, the electrophysiological signature of prestin, a double-point mutation...
(V499G/Y501H), was introduced that eliminates the NLC of prestin (26). The V499G/Y501H is located at or near the beginning of the COOH-terminal tail. This double mutation was previously shown to be properly targeted to the plasma membrane (26), which was confirmed in this study by the presence of fluorescence in TIRF mode. The V499G/Y501H mutants displayed a significant reduction in ΔF/F upon high K⁺ application compared with the wild-type prestin constructs (Fig. 4C). In the case of the COOH terminally labeled prestin, almost no intersubunit FRET changes were observed for the mutant upon application of high K⁺ concentration solution (Fig. 4, A and C). Furthermore, when COOH-terminally labeled mutant prestin was cotransfected with COOH-terminally labeled wild-type prestin in a 1:1 ratio, little to no FRET changes were observed (data not shown), suggesting that multiple subunits of prestin function in a highly concerted manner. The FRET changes were also significantly reduced in the case of intersubunit FRET between FlAsH-prestin (V499G/Y501H) and prestin (V499G/Y501H)-CFP (Fig. 4, B and C).

Sodium salicylate is a known ototoxicant that has been shown to reversibly block electromotility in outer hair cells (11, 24). It can eliminate NLC and reduce change movement in prestin-expressing mammalian cells by binding competitively at the Cl⁻ binding site (17). FRET efficiency changes between the COOH-termini were reversibly diminished in the presence of 10 mM sodium salicylate (Fig. 5). To exclude the possibility that this effect was a result of a sodium salicylate-induced change in fluorescence intensity due to modification of the local environment of the fluorophore (e.g., intracellular local pH), it was confirmed that the fluorescence intensity of CFP or YFP, in this case attached to the prestin molecule, did not change upon application of sodium salicylate. Taken together, these results strongly suggest that the changes in FRET efficiency we observe are indeed prestin-specific conformational changes related to NLC.

Decrease in FRET is observed under control of membrane potential by patch clamp. The characteristic feature of OHCs is the very fast cell-length change responding to membrane potential changes up to 20 kHz (4), and accordingly the conformational change of prestin is thought to be similarly fast. The FRET change we detected in this study is, however, slow as shown in Figs. 2–5. It is possible that the slowness is due to the rather slow change of the membrane potential when the bath was perfused with high K⁺ solution. To clarify this point and also to further confirm that the FRET change is a response to membrane potential change but not to high K⁺ solution, we carried out simultaneous recording of FRET under TIRF and whole cell patch clamp.

For this experiment, it was necessary to select patchable cells that are less flat and less firmly attached to the coverslips. The detection of a clear FRET change from these cells was difficult partly due to a lower fluorescence signal under TIRF. As a result, we first selected cells that show a clear decrease in FRET when high K⁺ solution was perfused (Fig. 6, A and B, black, Fig. 6C, left) (4 K⁺, 0.99 ± 0.005; 140 K⁺, 0.94 ± 0.015; n = 4, P < 0.01) and then achieved whole cell configuration by patch pipette. We then recorded the change of membrane potential under CC condition (Fig. 6A) and simultaneously monitored changes in FRET (Fig. 6B, red). After latency due to the perfusion of the bath solution, the membrane potential changed rather rapidly within a second, confirming that the high K⁺ solution effectively induced rapid membrane potential change. A significant decrease in FRET was observed (Fig. 6C, middle) (4 K⁺, 1.01 ± 0.008; 140 K⁺, 0.96 ± 0.011; n = 4, P < 0.01), but the time course of FRET decrease was very slow (Fig. 6B, red). Finally, we recorded from the same cell under VC to achieve rapid and precise control of membrane potential without changing the bath solution. Following the results of CC condition, depolarizing step pulse from −80 to 0 mV was applied (Fig. 6A), and the FRET change was...
recorded. A significant change of FRET was observed with no
bath solution change (Fig. 6C, right) (−80 mV, 1.01 ± 0.011; 140 K+, 0.94 ± 0.022; n = 4, P < 0.05), confirming that the
responses in previous experiments are truly due to membrane
potential change. However, the time course was as slow as the
cases of control and CC condition (Fig. 6B, blue).

The NLC peaks at around −50 mV showing voltage-depen-
dent conformational change peaks at −50 mV (Fig. 1). To
better understand the correlation between NLC and the FRET
changes in the present study, we compared the FRET values at
−120, −70, −20, +30, and then −120 mV, which are all
separated by 50 mV. The obtained FRET values at each
membrane potential are plotted in Fig. 7 (−120 mV, 1.01 ±
0.007; −70 mV, 0.99 ± 0.009; −20 mV, 0.96 ± 0.022;
+30 mV, 0.96 ± 0.017; −120 mV, 1.00 ± 0.007; n = 8). The
values at −20 mV and +30 mV, but not at −70 mV, were
significantly (P < 0.05) smaller than that at −120 mV. This
result is consistent with the voltage dependency of NLC shown
in Fig. 1, further supporting the link between NLC and the
FRET change.

Fig. 5. Effect of sodium salicylate on FRET changes. A: FRET changes are
reversibly reduced in the presence of 10 mM sodium salicylate, applied
between 300 and 600 s (vertical dotted lines), n = 10. Average nF/I
CFP = 0.22 ± 0.02. B: ΔF/F value during coapplication of 140 mM KCl and 10 mM
sodium salicylate (NaSal) (0.086 ± 0.007) is significantly reduced compared
with the FRET changes observed upon high KCl application before and after
sodium salicylate application (ΔF/F = 0.10 ± 0.009 and 0.12 ± 0.01,
respectively). n = 10, P < 0.0001.

Fig. 4. FRET changes are related to the NLC of prestin. Data are normalized
as indicated in Fig. 2. Bars indicate application of 140 mM KCl. A: FRET
changes observed between the COOH-termini of wild-type XFP-labeled pres-
tin (prestin-CFP and Prestin-YFP, n = 16, open circles) and prestin expressing
the V499G/Y501H NLC-elimination double-point mutation (PrestinV499G/
Y501H-CFP and PrestinV499G/Y501H-YFP, n = 14, open squares). Average
nF/I CFP for the construct bearing the double-point mutation was 0.14 ±
0.02. B: intersubunit FRET changes between the NH2- and COOH-termini of prestin
expressing the V499G/Y501H NLC-elimination double-point mutation (FLAsH-PrestinV499G/Y501H and PrestinV499G/Y501H-CFP, n = 6, open squares) and fluorescently labeled wild-type prestin (FLAsH-prestin and pres-
tin-CFP, n = 5, open circles). Average nF/I CFP for the construct bearing the
double-point mutation was 0.8 ± 0.1. C: NLC-eliminating double-point
mutation significantly reduced the change in FRET (ΔF/F) observed between
the COOH-termini and the NH2- and COOH-termini of prestin. The P values
were obtained from an unpaired t-test.
DISCUSSION

The molecular mechanisms by which prestin senses changes in voltage and transduces these changes into a structural rearrangement are currently unknown. Using a TIRF/FRET approach, we were able to study the conformational changes of prestin that occur upon membrane depolarization. These results constitute a first glimpse into the molecular movements that underlie normal prestin function. They also further confirm that prestin operates as a functional oligomer and reveal some unanticipated aspects of prestin motion in response to changes in membrane potential.

Observed FRET changes imply an increase in the distance between fluorescently labeled prestin subunits upon depolarization. Changes in FRET values are attributable to either a change in the distance between acceptor and donor fluorophores, a change in the relative orientation of acceptor and donor fluorophores, or a change in the local environment of one or both of the fluorophores. The observed FRET changes are unlikely to be a result of this last option, because neither the mutant V499G/Y501H nor mGluR display any change in fluorescent properties due to high K+ solution application. Therefore, the observed FRET changes can be confidently attributed to actual changes in prestin structure.

OHCs decrease and increase in length upon membrane depolarization and hyperpolarization, respectively (3). This morphological change is presumed to be related to the molecular rearrangements of prestin, meaning that the surface area of prestin would increase as a result of hyperpolarization and decrease during depolarization. One might expect that this would lead to an increase in FRET efficiency (decrease in distance) for fluorophores attached at the NH2- and COOH-termini of prestin, both within a single prestin subunit and among subunits of a multimer. However, decreases in FRET were observed upon application of depolarizing concentrations of KCl, indicating a potential increase in the distance and/or the angle between fluorophores. The similarity in the FRET changes observed in all three labeling combinations suggests that the observed FRET changes may be a result of a common structural rearrangement. Since prestin is labeled at the COOH-terminus in all cases, a likely explanation is that the FRET changes reflect a rearrangement in the COOH-termini of prestin upon membrane depolarization. Previous studies have demonstrated the importance of the NH2 and COOH-termini in prestin function (12, 15, 26), but the nature of their involvement is still unknown, owing to the lack of structural and dynamic data on prestin. The struc-

Fig. 6. Simultaneous recording of patch clamp and TIRF-FRET. A: TIRF-FRET measurement was done as in previous figures (control). CC, measured cell was recorded by whole cell-patch clamp, and the membrane potential change during 140 mM KCl application was recorded under current clamp condition. VC, membrane potential of the same cell was controlled by voltage clamp with no bath solution exchange. B: time-lapse change of normalized nFl/CFP under control (black), current clamp (red), and voltage clamp (blue) condition. C: normalized nFl/CFP values before, during, and after 140 mM KCl application (Control, CC) or holding at 0 mV under VC (n = 4, respectively). *P < 0.05, and **P < 0.01.

Fig. 7. Voltage-range dependency of TIRF-FRET change. A: time-lapse change of normalized nFl/CFP under voltage-clamp. Average and SE (n = 8) are shown. Normalized nFl/CFP values at −120, −70, −20, +30 mV, and then −120 mV, each separated by 50 mV, were recorded. B: normalized nFl/CFP values under voltage clamp at the holding potentials of −120, −70, −20, +30, and −120 mV (n = 8). The nFl/CFP for the final 30 s at each potential were averaged, and the averaged nFl/CFP was normalized by baseline nFl/CFP. Statistical analysis was carried out by Student’s t-test. *P < 0.05; n.s., not significant.
tural rearrangements reported here may provide some clue as to how the intracellular termini are contributing to prestin’s function.

**Observed FRET changes imply a slow conformational change in HEK cells.** The changes in FRET efficiency upon high KCl application occur far more slowly than the conformational changes of prestin are presumed to take place in OHCs. The electromotile response in OHCs occurs on the order of less than one millisecond (4). There are several possibilities for this apparent discrepancy. First, part of the slowness we observe owes to the speed of the fluidics perfusion system. However, the delay due to fluidics was shown to be minor, because the membrane potential change recorded under current clamp condition was much faster than the speed of FRET change (Fig. 6A). Likewise, the time course of FRET changes were similar regardless of whether the membrane potential was rapidly changed under voltage clamp or by application of high KCl solution (Fig. 6B).

A second possibility is that endogenous K⁺/Cl⁻ transporters are depleting intracellular chloride stores in response to the intracellular chloride concentration. In this scenario, the observed FRET changes may be related to the way in which prestin uses intracellular chloride to sense changes in membrane potential. Thus the slow FRET change we observed might be a result of a change of intracellular Cl⁻ concentration upon high K⁺ application. However, this is unlikely because a similar FRET change was observed even when the intracellular Cl⁻ concentration was maintained constant by patch pipette (Fig. 6).

Third, the TIRF/FRET experimental setup may be partially responsible for the slowness of the observed fluorescence changes. NLC recordings of heterologously expressed prestin typically require that the cells be loosely attached to the glass coverslip (6). To facilitate the TIRF experiments, recordings were obtained from attached cells and their adhesion to the coverslip might have influenced the speed of FRET change. A related possibility is that the observed FRET change might be influenced by differences in the cellular scaffolding in HEK293T cells versus the native OHCs. In fact, differences in motility between prestin-expressing TSA201 cells and native OHCs are well established. Observing motile responses in prestin-expressing TSA201 cells required physical distortion of the spherical cells by drawing them into a suction pipette (28). Taken together, the extent and speed of FRET change in nonadhered transfected HEK293 cells or in OHC cells might be expected to differ somewhat from those in the adhered HEK293 cells used in the present study. However, it is unlikely that these anticipated differences could fully account for the observed slow rate of FRET change relative to the much faster rate of electromotility, as the speed of FRET change remained slow in patchable, loosely adhered cells used for simultaneous recording of patch clamp and TIRF-FRET (Fig. 6).

Finally, and perhaps most likely, the slow FRET change might reflect a novel slow conformational change of prestin distinct from the reported rapid one, which is indirectly related, but clearly linked, to NLC. Further studies are needed to clarify the specific underlying molecular mechanisms of the observed FRET changes.

Regardless of the exact molecular nature of the observed FRET changes, the observed conformational changes are related to NLC, the electrophysiological signature of prestin. However, this link may be indirect, as their kinetics remarkably differ each other. The V499G/Y501H double point mutation has been demonstrated to eliminate NLC in heterologously expressed prestin (26). Our TIRF/FRET data indicate no or diminished structural rearrangements when this mutation is present (Fig. 4). Sodium salicylate, which reversibly blocks NLC both in OHCs (11, 24) and in prestin-expressing mammalian cells (17), also reversibly reduces the observed FRET changes in response to membrane depolarization (Fig. 5). Both the sodium salicylate and double point mutation data provide strong evidence that eliminating NLC alters the dynamic behavior of prestin, as evidenced by the elimination or dramatic reduction in FRET changes. This connects the movements of the intracellular termini with the voltage-sensing ability of prestin, as previously suggested by mutagenesis studies (26).

These results constitute the first dynamic data of the molecular rearrangements of prestin. Identification of structural elements underlying prestin function will hopefully lead to a better understanding of the molecular mechanisms that underlie mammalian hearing.

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