Probing Conformational Changes of Prestin with Thiol-Reactive Optical Switches

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ABSTRACT Thiol-reactive optical switch probes were used to examine conformational changes of prestin-based membrane motor. Because this motor is based on mechanoelectric coupling similar to piezoelectricity, the motile activity can be monitored by charge movements across the plasma membrane, which appears as nonlinear capacitance. When the plasma membrane is conjugated with the probes, optically induced spiro-merocyanine transition positively shifted nonlinear capacitance of outer hair cells and prestin-transfected cells by ~10 mV. These shifts were reversible and were eliminated by pretreatment with iodoacetamide. However, they were little affected by pretreatment with biotin maleimide, which cannot reach the cytoplasmic surface. Our results showed that merocyanine states, with a larger dipole moment, interact with the motor's extended conformation stronger than with the compact conformation by 1.6×10^{-21} J/molecule. The interaction sites are near the cytoplasmic side of the motor protein.

INTRODUCTION

The membrane motor of outer hair cells, which is essential for the sensitivity and frequency selectivity of the mammalian ear (1), uses electrical energy associated with the receptor potential available at the plasma membrane. Electric energy is converted into mechanical energy by electromechanical coupling (2–6). Specifically, the motile elements have conformational states differing in charge and the membrane area, and charge transfer induced by changes in the membrane potential is coupled with changes in the membrane area, displacing the cell (7,8). Movement of this charge is observed as nonlinear capacitance (2,9,10).

This motile activity, which is called electromotility, is based on prestin (11), a member of the SLC26 family of anion transporters. Although conformational changes in prestin involve changes of the membrane area coupled with charge movement, little is known about molecular details. For this reason, probes that interact with charges of the membrane protein are of considerable interest.

Thiol-reactive spirobenzopyrans bind to cysteine groups of proteins, and upon ultraviolet-irradiation turn into their merocyanine conformations, which have dipole moments four times larger than their respective spiro conformations (12). Irradiation of green light accelerates these probes' return to their spiro conformations. These molecules, known as optical switches, could therefore serve as useful probes of charge displacements near cysteine groups associated with conformational changes in prestin.

If one conformational state of prestin has larger dipole moments near one or some of the cysteine groups, that state is favored because interaction with the merocyanine state of the optical probe reduces the relative free energy of that state. Such an effect can be observed as voltage shifts in nonlinear capacitance.

Here we report the effect of optical switches on prestin in outer hair cells and in transfected cells. Specifically, we examine how these molecules affect nonlinear capacitance due to prestin by monitoring the membrane capacitance.

MATERIALS AND METHODS

Media

Our standard external medium contained 135 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 5 mM Na-HEPES with pH adjusted to 7.4. The osmolarity was adjusted to 300 mOsm/kg by glucose.

The external blocking medium that we used consisted of 135 mM NaCl, 5 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $CoCl_2$, 10 mM Na-HEPES, and ~10 mM glucose, which was used to adjust the osmolarity to 300 mOsm/kg. The pH was adjusted to 7.4.

The intracellular blocking medium was made of 140 mM CsCl, 2 mM $CaCl_2$, 5 mM EGTA, 10 mM K-HEPES, pH 7.4.

Cell preparation

Isolated outer hair cells (OHCs) were prepared in a manner described in Dong et al. (13). Briefly, bullas were harvested from guinea pigs in accordance with our animal protocol (1061-02 NINDS/NIDCD). The organ of Corti was dissociated from opened cochleas by teasing with a fine needle under a dissection microscope. These Corti strips were collected for treatment with the optical switch reagents.

Prestin-transfected cells were prepared following the method described in Dong and Iwasa (14). Human embryonic kidney (HEK) 293T cells were cultured in 35-mm dishes filled with Dulbecco's Modified Eagle Medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The cells were cotransfected with prestin-encoding plasmid pcDNA3.1 and green fluorescent protein-encoding plasmid. We used Lipofectamine 2000 (Invitrogen) as the transfection reagent. Approximately 90% of the cells that expressed green fluorescent protein had voltage-dependent capacitance, the indicator of prestin in the plasma mem-

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brane. These cells, between 24 and 48 h after prestin transfection, were washed by the external medium for treatment with the optical switch reagents.

Treatment with optical switch reagents

Five thiol-reactive optical switch probes, Nitro-BIPS-5′-C6Mal, Nitro-BIPS-5′-C6Mal-8-I, Nitro-BIPS-8,6′-DI, Nitro-BIPS-4′-Br, and Nitro-BIPS-8-I (Fig. 1) were synthesized at the University of Wisconsin as described in Sakata et al. (12). These probes were stored at −20°C until use. Optical probes were dissolved in DMSO at 20 mM and then diluted to 50 μM or 20 μM into the external blocking medium. The DMSO concentration used for incubation was 0.25%.

Strips of Corti's organ were incubated in a solution that contained one of the optical switch reagents for 60–80 min at room temperature. They were then rinsed with the regular external medium. Outer hair cells (OHCs) were isolated by gently triturating these strips for three times with a plastic pipette. Isolated OHCs were placed in a chamber mounted on an inverted microscope. The length of the cells used for patch-clamp recoding ranged between 40 and 75 μm.

Transfected cells were rinsed with the standard external medium and then incubated in a solution that contained one of the optical probes at 20 μM for 60–80 min at room temperature. These cells incubated with 0.05% trypsin-EDTA solution (Invitrogen) for 5–10 min at 37°C after the probe solutions were removed. Then, these cells were washed by external blocking medium and collected for patch-clamp experiments.

Preincubation with cysteine blockers

To block the binding of the optical probes to prestin, pretreatments by iodoacetamide (IAM) and biotin maleimide (BM) (purchased from Sigma, St Louis, MO) were attempted on some of the cells. Iodoacetamide was dissolved into the external blocking medium at 200 μM concentration. Biotin maleimide was dissolved in DMSO at 200 mM and then diluted into the external blocking medium at 2 mM or 500 μM. DMSO concentration in the final solution was 1%.

The preincubation with cysteine blocker was done on OHCs before dissociation from the organ of Corti. The duration was 30 min for IAM and 60 min for BM at room temperature (23°C).

Electrophysiological recording

Electrophysiological recording was performed using an inverted microscope (Diaphot 300, Nikon, Tokyo, Japan) in combination with a micromanipulator (Newport, Newport Beach, CA), The patch pipette was mounted on the micromanipulator associated with the head stage of the patch amplifier (Axopatch 200B, Axon Instruments, Barrington, NJ). Voltage waveforms were generated with an ITC-16 interface (Instrutech, Fort Washington, NY) in combination with a Macintosh computer running the IGOR program (WaveMetrics, Lake Oswego, OR) with Pulse Control XOPs (data acquisition modules from Instrutech). The patch pipettes were made from borosilicate capillaries (OD 1.5 mm, ID 1.1 mm, Sutter Instrument, Novato, CA) with a pipette puller (Model P-97, Sutter Instrument) and used without fire-polishing.

To facilitate membrane capacitance measurement, the channel blocking media described earlier were used for experiments. The pipette resistance was between 2.5 and 4.5 MΩ in the bath. In the whole-cell configuration, the access resistance R_a was between 5 and 8 MΩ. The membrane resistance R_m was somewhat dependent on the membrane potential and was between 200 and 800 MΩ.

Membrane currents were recorded in the whole-cell configuration. The membrane capacitance was determined from capacitive currents elicited by a staircase voltage waveform ascended from −130 mV to +110 mV in 10 mV steps. The sampling interval of the data acquisition was 10 μs. To concisely describe the bell-shaped voltage dependence of the capacitance we fit our data with a function

$$C_m(V) = C_{lin} + 4C_{max}\frac{B(V)}{(1+B(V))^2} \quad (1)$$

with

$$B(V) = \exp\left[q(V - V_{1/2})/k_BT\right]. \quad (2)$$

This function has a peak value $C_{max} + C_{lin}$ when membrane potential V is $V_{1/2}$. The sharpness of the peak is determined by charge q. The quantities k_B and T are, respectively, Boltzmann's constant and the temperature. Equation 1 is consistent with a two-state model in which transition between its two states is accompanied by transfer of charge q across the membrane.

Turning optical switches

Each optical probe conjugated with proteins was initially in its spiro conformation. To convert it into the merocyanine conformation, the specimens were irradiated with ultraviolet (UV) light, from the fluorescence light source of the microscope, which had an HBO 10-W mercury short-arc photo-optical lamp, through a 365-nm interference UV filter (Edmund Optics, Barrington, NJ). The exposure time was between 30 and 120 s. For switching from the merocyanine conformations back to the spiro conformations, the samples were irradiated with light filtered with a 546-nm interference filter (Edmund Optics). The exposure time was 1–15 min.

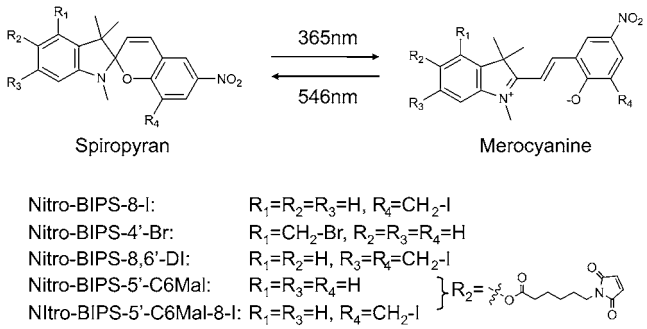

FIGURE 1 Nitro-BIPS optical switches and their light-induced transitions.

RESULTS

Nonlinear capacitance of OHCs

Optical switches Nitro-BIPS-8-I and Nitro-BIPS-4′-Br were used for initial experiments on OHCs.

The peak capacitance of OHCs treated with the optical switch reagents was between −25 and −60 mV, approximately the same as untreated cells. After a brief exposure to UV light that induces spiro-merocyanine transition, the membrane capacitance showed a positive shift of ~10 mV (Figs. 2 *A* and 3 *A*).

The shift was reverted by exposure to green light if the exposure to UV light was up to 80 s (Fig. 2 *B*). The exposure time to green light for recovery was much longer, typically

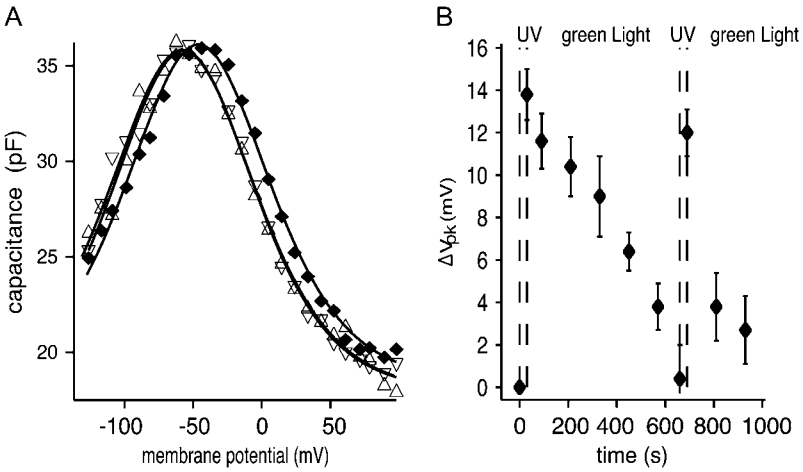

FIGURE 2 Effect of the optical switch Nitro-BIPS-8-I on voltage-dependent capacitance of outer hair cells. The cells were treated with 50 μM optical switch reagent. (*A*) Voltage shift of nonlinear capacitance induced by UV irradiation. The membrane capacitance is plotted against the membrane potential. Control (Δ), after 30 s UV exposure ($\blacklozenge$), and recovery with green light (∇). Parameter values for the fit (mean $\pm$ SD): control, $q = (0.75 \pm 0.03)e$; $C_{lin} = (17.9 \pm 0.5)$ pF; $V_{1/2} = (-56.8 \pm 1.0)$ mV; and $C_{max} = (17.8 \pm 0.5)$ pF. UV exposure, $q = (0.75 \pm 0.02)e$; $C_{lin} = (18.4 \pm 0.4)$ pF; $V_{1/2} = (-46.2 \pm 0.7)$ mV; and $C_{max} = (17.7 \pm 0.4)$ pF. Recovery, $q = (0.74 \pm 0.02)e$; $C_{lin} = (17.9 \pm 0.4)$ pF; $V_{1/2} = (-57.8 \pm 0.8)$ mV; and $C_{max} = (17.9 \pm 0.3)$ pF. (*B*) Reversibility of voltage shifts. Error bars indicate standard deviations.

~10 min for full recovery of nonlinear capacitance (Fig. 3 *B*). No recovery was recognized without the exposure to green light for ~15 min of recording.

During the course of the experiment, we noticed that the recovery (observed as negative shifts of nonlinear capacitance with green light exposure) was prevented if the bathing medium was allowed to become hyperosmotic by evaporation. Since hyperosmotic media induces negative voltage shifts (2), the effect of the optical probes is clearly distinguished from the effect of membrane pressure changes due to hyperosmotic medium.

Reversibility

A prolonged exposure to UV light beyond 80 s induced a larger positive shifts up to ~25 mV in the voltage dependence accompanied by a decrease in the peak capacitance. The total charge transfer also decreased. Green light irradiation was not effective to induce recovery from this state (not shown).

As a control, OHCs not treated with the optical switch reagents were irradiated with the UV light. After 3 min of irradiation, nonlinear capacitance started to show positive shifts in the voltage dependence and peak height started to decrease.

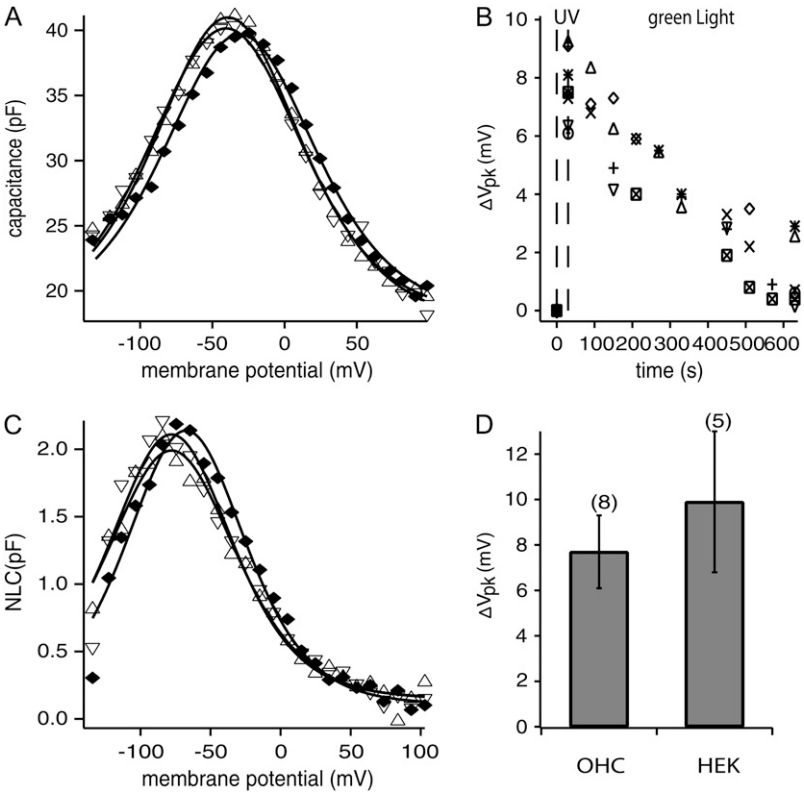

FIGURE 3 Effect of optical switch Nitro-BIPS-4-Br on voltage-dependent capacitance. The cells were treated with the optical switch reagent at 20 μM. (*A*) The membrane capacitance of OHC. Control ($\triangle$), after 30 s exposure to UV ($\blacklozenge$), and recovery with green light (∇). Parameter values for the fit are, control: $q = (0.78 \pm 0.03)e$; $C_{lin} = (18.2 \pm 0.5)$ pF; $V_{1/2} = (-39.2 \pm 0.7)$ mV; and $C_{max} = (21.6 \pm 0.7)$ pF. UV exposure: $q = (0.75 \pm 0.04)e$; $C_{lin} = (18.1 \pm 0.8)$ pF; $V_{1/2} = (-31.0 \pm 1.0)$ mV; and $C_{max} = (22.8 \pm 0.5)$ pF. Recovery: $q = (0.72 \pm 0.03)e$; $C_{lin} = (17.3 \pm 0.6)$ pF; $V_{1/2} = (-40.6 \pm 0.8)$ mV; and $C_{max} = (22.8 \pm 0.6)$ pF. (*B*) Time course of optical switching on OHCs. The cells are exposed to UV light during the initial 30 s from time 0. Then the cells are exposed to green light. (*C*) The nonlinear capacitance of prestin-transfected HEK cell. Control ($\triangle$), 30 s UV exposure ($\blacklozenge$), recovery with by green light (∇). Parameter values for the fit. Control: $q = (0.85 \pm 0.04)e$; $C_{lin} = (0.15 \pm 0.04)$ pF; $V_{1/2} = (-77.8 \pm 1.4)$ mV; and $C_{max} = (1.85 \pm 0.05)$ pF. UV exposure: $q = (0.92 \pm 0.05)e$; $C_{lin} = (0.10 \pm 0.05)$ pF; $V_{1/2}k = (-67.5 \pm 1.4)$ mV; and $C_{max} = (2.03 \pm 0.07)$ pF. Recovery: $q = (0.89 \pm 0.06)e$; $C_{lin} = (0.15 \pm 0.06)$ pF; $V_{1/2} = (-78.0 \pm 1.8)$ mV; and $C_{max} = (1.96 \pm 0.08)$ pF. (*D*) Voltage shifts of nonlinear capacitance induced by UV light in OHCs and in prestin-transected HEK treated with Nitro-BIPS-4-Br. The duration of exposure is 30 s for both. Error bars indicate standard deviations. The numbers indicate the sample size *N*.

Prestin-transfected cells

To examine whether the voltage shifts observed for OHCs are based on prestin, the experiment was repeated with prestin-transfected cells (Fig. 3 *C*). The shifts in nonlinear capacitance were similar to those observed for OHCs (Fig. 3 *D*). This result is consistent with the assumption that the effects of the optical probes observed for OHCs are due to prestin.

Pretreatment of cysteine groups

We attempted to prevent some or all conjugation of membrane proteins with the optical probes by pretreating the cells with thiol reactive reagents. One such reagent was iodoacetamide, which can penetrate the plasma membrane. Another one was biotin maleimide, which cannot reach the cytosolic surface.

Iodoacetamide

The pretreatment by iodoacetamide eliminated light-induced voltage shifts of nonlinear capacitance of OHCs (Fig. 4). This observation indicated that iodoacetamide pretreatment made cysteines unavailable for conjugation with the optical probes.

Biotin maleimide

In contrast, the cells pretreated with biotin maleimide showed sensitivity to light exposure indistinguishable from the cells which were not pretreated (Fig. 4). This observation implies that the optical groups that are conjugated with prestin at a cysteine residue or residues in the cytosolic side are responsible for their effect on nonlinear capacitance.

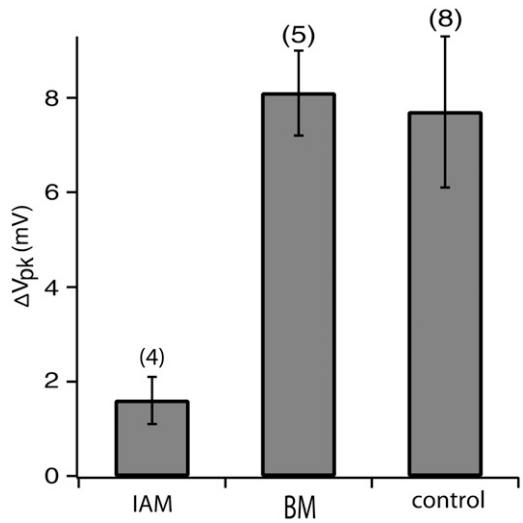

FIGURE 4 Effects of preincubation iodoacetamide (IAM) and biotin maleimide (BM). IAM is membrane-penetrable, whereas BM cannot reach the cytosolic side of the plasma membrane. The optical switch is Nitro-BIPS-4-Br conjugated at 20 μM. Error bars and the numbers respectively indicate standard deviations and the sample size *N*.

Comparison of optical switches

We compared five optical switches for the effectiveness in inducing voltage shifts (Table 1). Although the most effective switches were Nitro BIPS-5′C6Mal and Nitro-BIPS-5′-C6Mal-8-I, which have the largest R2 residues, the differences are not large.

DISCUSSION

Previous reports show that thiol reagents may have effects on electromotility (15–17). Those reports showed that the significance of the effects highly depends on the reagent. The preparation used in this article differed from those reports in that the cells treated with the optical probe reagents were thoroughly washed before measurements, leaving only those optical probe molecules that were conjugated with proteins. The quantities that characterize nonlinear capacitance of our OHC preparations before UV irradiation were similar to those of nontreated cells (18). Specifically, the motor charge q was $\sim$0.8e, the peak nonlinear capacitance C_{max} was $\sim$20 pF, and the peak potential $V_{1/2}$ was between -30 and -50 mV (see Figs. 2 *A* and 3 *A*). In addition, our observations were a brief UV irradiation and a longer exposure to green light, which affect the conformations of the optical probes.

The optical probes that we used were not specific to prestin. They should bind equally well to other membrane proteins with cysteine groups. However, the effect of these probes' conformational changes on nonlinear capacitance must be due to their direct interaction with prestin molecule rather than an indirect effect mediated by other membrane proteins, for two reasons. First, the recovery from the positive shifts of the voltage dependence associated with spiro- to merocyanine transition of the probes was inhibited in hyperosmotic media, which has the effect of bringing about negative shifts in voltage dependence. This observation rules out the possibility that the observed positive shift was associated with an increase in membrane tension as an indirect effect of the conformational change of the optical probes through other proteins. Second, the voltage shifts observed on prestin-transfected HEK cells were similar to those in OHCs. Because the structure of those transfected cells differ significantly from OHCs (14), it is hard to seek common

TABLE 1 Effectiveness of optical switches

Optical probe	Shift (mV)	N
Nitro BIPS-8-I	6.9 ± 1.3	6
Nitro BIPS-4′-Br	7.7 ± 1.6	8
Nitro BIPS-5′-C6Mal	12.7 ± 2.1	5
Nitro BIPS-8,6′-DI	9.3 ± 2.1	3
Nitro BIPS-5′-C6Mal-8-I	11.0 ± 2.6	3

Voltage shifts (mean shifts ±SD) induced by optical probes are compared. Outer hair cells were treated with each 20 μM optical switch reagents. UV exposure was for 30 s. See Materials and Methods for details.

factors that results in the voltage shifts in nonlinear capacitance except for the probes' direct effect on prestin.

Implications on conformational changes of prestin

We have observed that the spiro-merocyanine transition of the optical probes elicited by UV light reversibly shifts the voltage dependence of prestin's nonlinear capacitance in the positive direction by ~10 mV. This shift implies that merocyanine conformation of the optical probes makes the extended state of prestin energetically more favorable over the compact state (19). The fact that the merocyanine conformation of each optical probes has a dipole moment four times larger than the spiro conformation indicates the significance of dipole interaction of the probes with the extended state of prestin (Fig. 5). This reduces the free energy of the extended state compared with the compact state, thereby favoring the extended state of prestin (19). Since the transfer of the electric charge between the two conformations of prestin is ~1 electronic charge, the difference in the interaction energy is ~10 meV/molecule (or 1.6×10^{-21}J/molecule).

Our observation that pretreatment with biotin maleimide did not affect the voltage shifts further indicates that the switch molecules that affect the conformational changes of prestin must be conjugated to cysteines on the cytosolic side of the molecule. The two models for prestin currently available indicate several candidates for such conjugation sites (Fig. 6). Those candidate sites also depend on the model. For this reason, it is not possible to suggest a specific site where the optical probes interact with prestin. However, it is certain that the interacting site is on the cytosolic side. This observation is interesting in view of the model proposed for the charge, which is coupled with conformational changes of prestin.

Since prestin transfers electric charge (motor charge) which is associated with its motile activity, considerable efforts has been made to identify the nature of this charge. The models proposed include both intrinsic charge and anions (20–22). All three models indicate changes in electric environment on the cytosolic side of prestin associated with conformational changes. For this reason, it could be important to examine whether our optical probes interact directly with the electrical environment associated with transfer of motor charge.

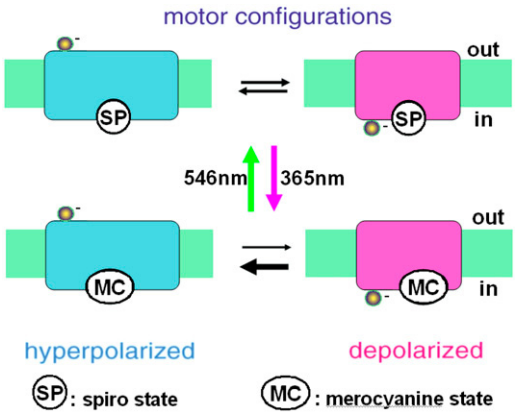

FIGURE 5 Schematic representation of an optical probe's effect on the membrane motor. The merocyanine conformation of the optical probe with a larger dipole moments stabilizes the extended state of prestin.

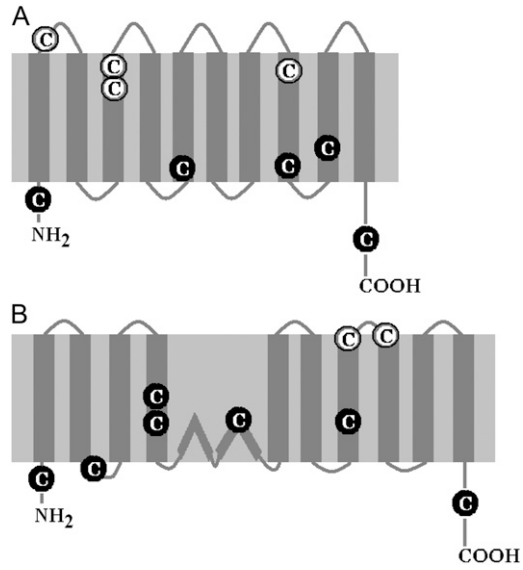

FIGURE 6 Putative locations of conjugation sites in prestin. Cysteine residues which are likely treated by biotin maleimide are marked with white background and those residues unlikely treated by BM are marked with black background. This assignment differs depending on the topological structure proposed by Navaratnam et al. (24) (*A*) or topological structure proposed by Déak et al. (25) (*B*).

Recovery time

The time courses for the voltage shifts with UV irradiation and the recovery with irradiation of green light are significantly longer than the spiro- and merocyanine conformational changes of unconjugated optical probes. The difference is quite striking for the time course for the recovery of nonlinear capacitance and the time course of merocyanine-spiro transition of unconjugated probes.

These differences in the time course can be understood as the result of raised free energy barrier to conformational changes of the optical probes by their conjugation with the protein. Indeed, increased transition times have been observed for those optical probes conjugated to actin although the difference is not as large (12).

Therefore, we can interpret that the positive shift of the voltage dependence associated with UV irradiation is due to the conformational transition of the optical probes from their spiro conformations to their respective merocyanine conformations.

Reversibility

Multiple sites

In this system, the effects of the optical switches on nonlinear capacitance do not appear as a clear on-off transition but a graded one. The UV exposure time of typically 30 s used for the experiments was shorter than the time constant of ~3 min. for voltage shifts. This may be related to the conjugation of optical switches to multiple sites of prestin, which has nine cysteines. Even though pretreatment by biotin maleimide would have reduced the number of cysteines that are relevant, this treatment still could leave multiple cysteine residues available for the optical switches. For this reason, we would expect that spiro-merocyanine transitions would proceed at multiple sites.

This factor could contributes to the irreversibility after a prolonged exposure to UV light because the involvement of multiple sites would make the recovery time beyond the time the whole-cell recording configuration could be maintained (up to 10 min.).

UV damage

Another factor for irreversibility can be UV damage. The effects of UV damage on untreated cells are positive voltage shifts of nonlinear capacitance and peak reduction, rather similar to the effects of spiro-merocyanine transition. By limiting the exposure time to <40 s, this effect was made <10% of the effect that was observed with optical probes. However, we cannot rule out the possibility that the presence of the probe molecules, which absorbs UV light, might have increased the susceptibility of the protein to UV light (23).

Effect of hyperosmotic medium

The recovery of voltage shifts was inhibited by hyperosmotic media. What is the implication of this observation? The voltage shifts accompanied by the transition from the spiro- to the merocyanine conformation of each optical switch indicates that the merocyanine conformation favors the extended state of prestin. Hyperosmotic media reduce membrane tension, making the extended state of prestin less favorable. These media therefore would elevate the energy level of the extended state-merocyanine complex and should facilitate the optical probe's transition to its spiro state. However, this expectation is the opposite of the observation. For this reason, we speculate that the factor that determines the recovery is the barrier and not the difference of free energy between these states. This issue would need further clarification.

CONCLUSIONS

We found that the optical probes induces positive shifts in nonlinear capacitance due to prestin. This shift indicates that the merocyanine states of the optical probes, which have larger dipole moments, have greater interaction with the extended state than the compact state of prestin. It is likely that the environment near cysteines in the cytosolic surface of prestin has a larger dipole moment in the extended conformation than in the compact conformation of prestin.

This method could be useful to study conformational changes of membrane proteins with fewer cysteine groups. To obtain more detailed information on membrane proteins with numerous cysteines, including prestin, a unique motor protein that has mechanoelectric coupling would be useful to combine this technique with molecular biological techniques. Specifically, these attempts should include reducing the number of cysteines to narrow down the location of the optical probe and focusing on a critical region by substituting another amino acid with a cysteine.

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