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SENSORY SYSTEMS

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Excitation of fluorescent dyes inactivates the outer hair cell integral membrane motor protein prestin and betrays its lateral mobility

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Abstract The outer hair cell motor protein, prestin, which resides exclusively in the cell's lateral membrane, underlies the mammal's exquisite sense of hearing. Here we show that photoexposure of the commonly used dyes Lucifer yellow, 6-carboxy-fluorescein, and 4-{2-[6-(dioctylamino)-2-naphthalenyl]ethenyl}-1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS), that are in contact with the cell's lateral membrane can photo-inactivate the motor irreversibly, as evidenced by reduction in prestin's gating charge displacement or non-linear capacitance. Furthermore, utilizing restricted fiber optic illumination of the lateral membrane, we show that whole-cell, non-linear capacitance is depleted beyond that expected for an immobile population in the exposed area. These data indicate that lateral diffusion of prestin occurs within the cell's lateral plasma membrane.

Keywords Lucifer yellow · Fluorescein · di-8-ANEPPS · Outer hair cell · FRAP · Gating charge · Membrane capacitance

Introduction

The molecular motors of the outer hair cell (OHC) drive a somatic mechanical response that underlies the enhanced sensitivity and frequency resolving power of the mammalian cochlea [2, 5, 12, 23]. Electrical measures indicate that these motors reside within the lateral plasma membrane of the OHC, and probably resemble other integral membrane proteins that are capable of generating gating currents upon voltage-induced conformational changes [3, 10, 11, 22]. In fact, Dallos and coworkers have cloned the gene for a protein, prestin, that, when expressed in non-auditory cells, presents electrical and mechanical signatures that match those of the OHC lateral membrane motor [25, 31]. Prestin is expressed in exactly that location, namely, the lateral plasma membrane [4], predicted by the electrophysiological evidence [10].

Integral membrane proteins are capable of diffusing through the plasma membrane [7]. To study the constraints that maintain prestin's residence in the lateral membrane, we have mapped recently the extent of prestin along the OHC lateral membrane using discrete mechanical stimulation [27], and evaluated the effect of cytoskeletal destruction on motor diffusion to areas normally devoid of motor molecules, namely, the basal region of the cylindrical cell. Surprisingly, the motors did not migrate to the basal region, indicating some type of integral membrane barrier. Here we use a technique to photo-inactivate motors while monitoring their gating charge movement so that their ability to diffuse within the lateral membrane can be assessed. We provide evidence that prestin, in intact OHCs, is capable of diffusing within the lateral plasma membrane.

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Materials and methods

Guinea pigs were euthanized with halothane. OHCs and supporting cells were isolated enzymatically with dispase I (0.5 mg/ml for 10 min followed by gentle trituration through a polyethylene pipette) in a modified Leibovitz medium containing (in mM): NaCl 142, KCl 5.37, $MgCl_2$ 1.47, HEPES 5, $CaCl_2$ 2 and dextrose 5; 300 mOsm, pH7.2. The cells were then transferred to a 700-µl perfusion chamber. All experiments were conducted at room temperature (~23 °C). A Nikon Diaphot inverted microscope with Hoffmann optics was used to observe the cells during electrical recording. All experiments were video-taped.

Single OHCs were studied under whole-cell voltage-clamp conditions using a patch clamp amplifier (200A, Axon Instruments, Union City, Calif., USA) at a holding potential of −80 mV. Initial resistances of patch pipettes were 2–3 MΩ, corresponding to tip sizes of 1–2 µm. Residual series resistance was 3–7 MΩ. Data were collected and analyzed using a Windows-based program (jClamp,

SciSoft, New Haven, Conn., USA). Ionic blocking solutions were used to remove voltage-dependent ionic conductances so that capacitive currents could be analyzed in isolation [10, 22]. The patch pipette solution contained (in mM): CsCl 140, $MgCl_2$ 2, EGTA 10, HEPES 5, with pH 7.2 and osmolarity 300 mOsm (adjusted with dextrose). The extracellular ionic blocking solution contained (in mM): 100 NaCl, 20 TEA, 20 CsCl, 1.25 $CoCl_2$, 1.48 $MgCl_2$, 10 HEPES, pH 7.2 and osmolarity 300 mOsm (adjusted with dextrose). Whole chamber perfusion was continuous.

OHC capacitance was monitored using transient analysis [10]. Additionally, continuous, high-resolution capacitance measurements were acquired through admittance analysis at time resolutions of 5.12 or 2.56 ms utilizing a two-sine wave voltage stimulus protocol fully described elsewhere [24]. The stimulus consisted of the sum of two voltage sine waves (390.625 and 781.25 Hz), each at a magnitude of 10 mV peak. In some cases, this AC stimulus was superimposed on voltage ramp or step stimuli. Capacitance-voltage (*C*-*V*) functions were fit to the first derivative of a two-state Boltzmann function relating non-linear charge to membrane voltage (d*Q*/d*V*; [10, 22]):

$$C_{\mathrm{m}} = Q_{max}\frac{ze}{kT}\frac{b}{(1+b)^2} + C_{\mathrm{lin}}$$

where

$$b = exp\left[\frac{-ze\left(V - V_{pkCm}\right)}{kT}\right]$$

and $Q_{\max}$ is the maximum nonlinear charge moved, V_{pkCm} the voltage at peak capacitance or, equivalently, at half-maximal nonlinear charge transfer, V_{m} is the membrane potential, z valence, C_{lin} linear membrane capacitance, e electron charge, k Boltzmann's constant, and T absolute temperature.

Patch pipettes were filled with either Lucifer Yellow (5%) or 6-carboxyfluorescein (5%) to load OHCs with the dye. The lipophilic dye 4-{2-[6-(dioctylamino)-2-naphthalenyl]ethenyl}-1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS) was delivered to the OHC plasma membrane as previously described [14, 15]. Briefly, cells were incubated with 1% dye in extracellular solution for 15 min prior to washing and recording. For whole-cell exposure, the dyes were excited with blue light (450–490 nm filter; Nikon 100-W high-pressure mercury lamp). For discrete excitation, a pulled quartz fiber optic (tip size ~2 μm) was fed directly from a Zeiss 75-W high-pressure mercury lamp, without filters. Whole cells were exposed by opening/closing the microscope light barrier manually. Fiber optic exposures were controlled via a computer-driven shutter.

Results

Prestin can be photo-inactivated

OHC membrane capacitance derives from two components: that associated with lateral membrane sensor/motor charge movement, and that proportional to membrane surface area. Figure 1 illustrates through measurements of capacitance that prestin can be irreversibly photo-inactivated. Figure 1a shows the exponential decrease in motor-associated capacitance following whole-cell photoexposure of a cell perfused intracellularly with 5% Lucifer Yellow via the patch pipette. The time constant for inactivation was 14.2 s (mean 26.4±10.2 s, n=6). To accomplish this inactivation, the photosensitive dye must be in contact with the cell's plasma membrane (Fig. 1b). In the absence of dye, photoexposure of OHCs did not reduce whole-cell capacitance; following perfusion of the cells with extracellular solutions containing fluorescent dye the motors became susceptible. The inactivation of lateral membrane motors was rapid and only occurred during the duration of the light exposure. This is shown in Fig. 1c where the light was switched on for only short periods; between exposures the reduced capacitance remained stable.

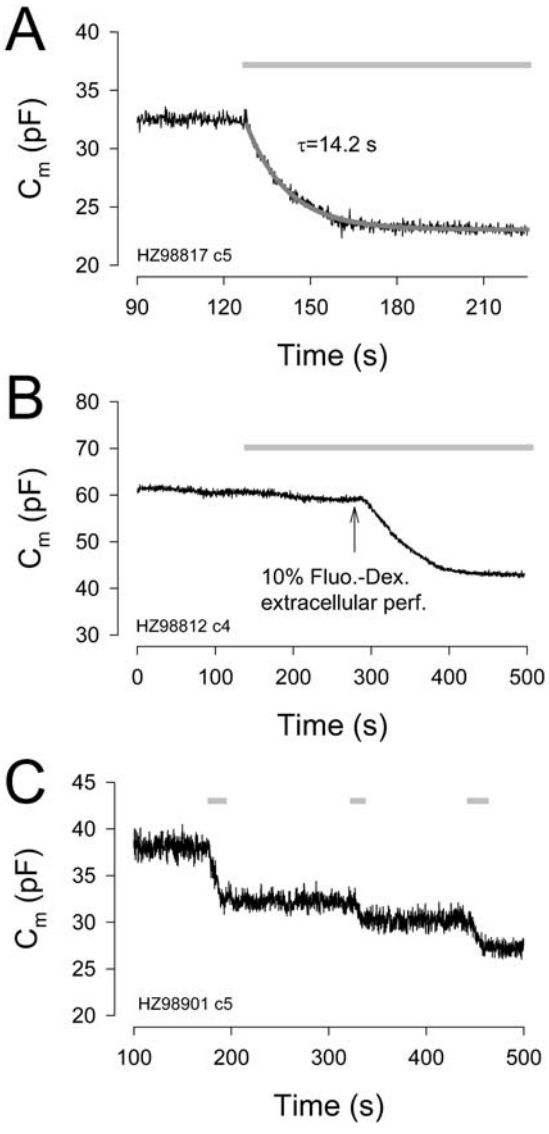

Fig. 1A–C Outer hair cell (OHC) motors can be irreversibly photo-inactivated. **A** OHC with intracellular Lucifer Yellow. Light was turned on for the period indicated by the *gray bar*. Note exponential decay of non-linear component of membrane capacitance (C_m, *ordinate*). Fit (*solid line*) indicates a time constant of 14.2 s. **B** Non-linear capacitance (NLC) was resistant to photoexposure in the absence of sensitizing dye. After the addition of the membrane impermeant dye fluorescein-dextran, NLC exponentially decreased. **C** OHC with 6-carboxyfluorescein intracellular. NLC decreased only during photoexposure (*bars*)

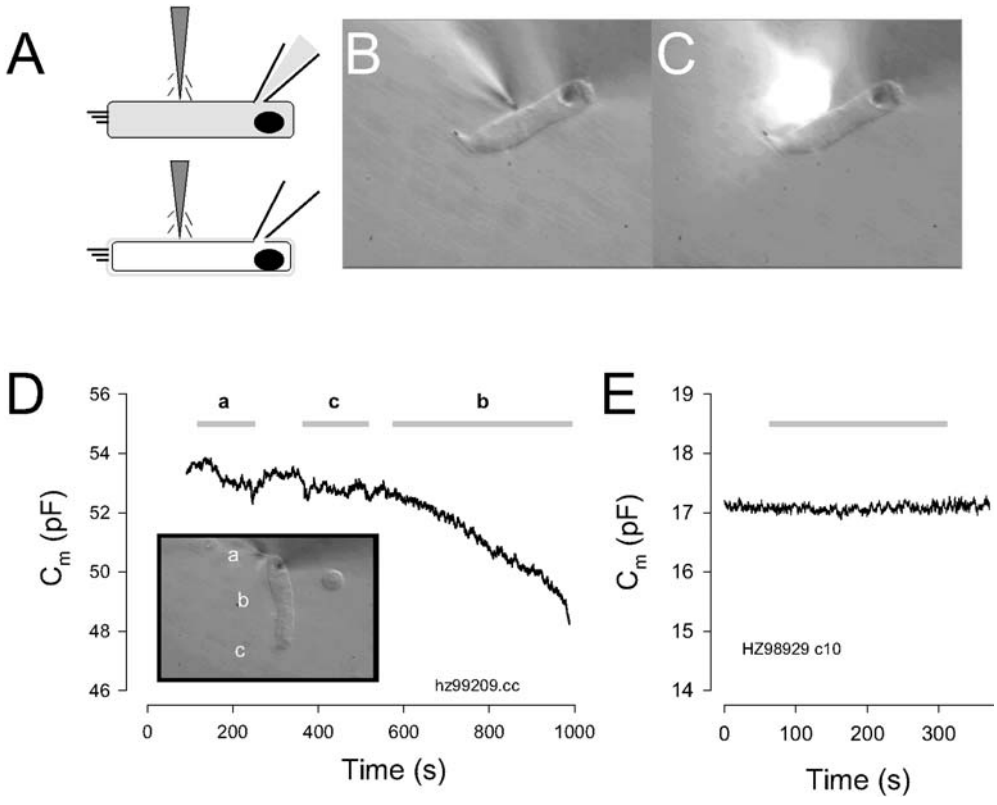

Fig. 2A–E Restricted exposure with fiber optic light probe. **A** Cells were whole-cell patch clamped and the fiber placed adjacent to the plasma membrane of cells with either intracellular dye (*top figure*, Lucifer Yellow) or intra-membrane [*bottom figure*, 4-{2-[6-(dioctylamino)-2-naphthalenyl]ethenyl}-1-(3-sulfopropyl)-pyridinium (di-8-ANEPPS) dye, see Methods]. **B**, **C** Digitally captured image of an OHC before and during exposure to the lateral membrane. **D** An OHC treated with di-8-ANEPPS was exposed at the basal (*a*), middle (*b*) and apical (*c*) regions of the cell. Only the lateral region exposure produced marked capacitance decreases. **E** A Deiters' cell treated with di-8-ANEPPS showed no reduction of linear capacitance during exposure

Photoexposure of restricted regions of the OHC

By delivering light through a tapered fiber optic light guide (tip size: ~2 μm; rough estimate of diffusive exposure: 80 μm^2), photo-exposure can be directed to selected regions of the OHC (Fig. 2). In Fig. 2d, exposure of the basal and apical region of the OHC produced little decrease in capacitance, whereas exposure of the lateral membrane caused a substantial drop in capacitance. This is expected, since the motors are restricted to the lateral membrane [4, 10]. In Deiters' cells, which lack prestin, membrane capacitance remained stable during photo exposure (Fig. 2e).

We used this restricted fiber optic exposure to evaluate the possibility of motor diffusion in the lateral membrane. Figure 3 illustrates that repeated or continuous restricted exposure of the OHC lateral membrane to the light source produced accumulating capacitance decreases not expected for an immobilized, finite population of motors. Repeated fiber optic exposure to fixed portions of the OHC sensitized with either intracellular 6-carboxy fluorescein (Fig. 3a), or with the membrane-soluble dye di-8-ANEPPS (Fig. 3b), resulted in an apparent linear (i.e. non-exponential) decrease in capacitance during exposure, and stabilization in the absence of exposure. The slope of the capacitance decrease with restricted exposures (12.3±1.8 fF/s; n=6) was substantially less than the initial slope (312±33 fF/s; n=6) following whole-cell exposure, though the ultimate magnitude of decrease could be quite large (Fig. 3c). We interpret these observations to result from the continuous movement of functional motors into the small region of photoexposure. That is, the rate of inactivation is limited by diffusion rate of the motor into the restricted region of exposure. Figure 4 shows whole-cell *C-V* functions for an OHC in which the lateral membrane was exposed for 8 min via fiber optic. In addition to a reduction in total charge moved (Q_{max}), indicating the removal of functional motors from the whole cell population, z and V_{pkCm} were altered.

Discussion

A number of studies have shown that integral membrane proteins are modified either directly by light at particular wavelengths [8, 9, 17] or by sensitizers following light activation [1, 17, 28]. Our data indicate that the OHC motor protein prestin can be rapidly photo-inactivated. Whole-cell *C-V* functions result from the combined characteristics of the photo-inactivated population of motors and the remaining normal populations of motors, and confirm the destruction of motors since Q_{max} was reduced. Motors are not directly susceptible to the light sources that we used but to some product that is released following the excitation of commonly used fluorescent dyes near or within the membrane. We have shown previously that gap junctions and K^+ channels within supporting Hensen's cells can be similarly photo-inactivated [20, 21]. It should be stressed that the decrease in

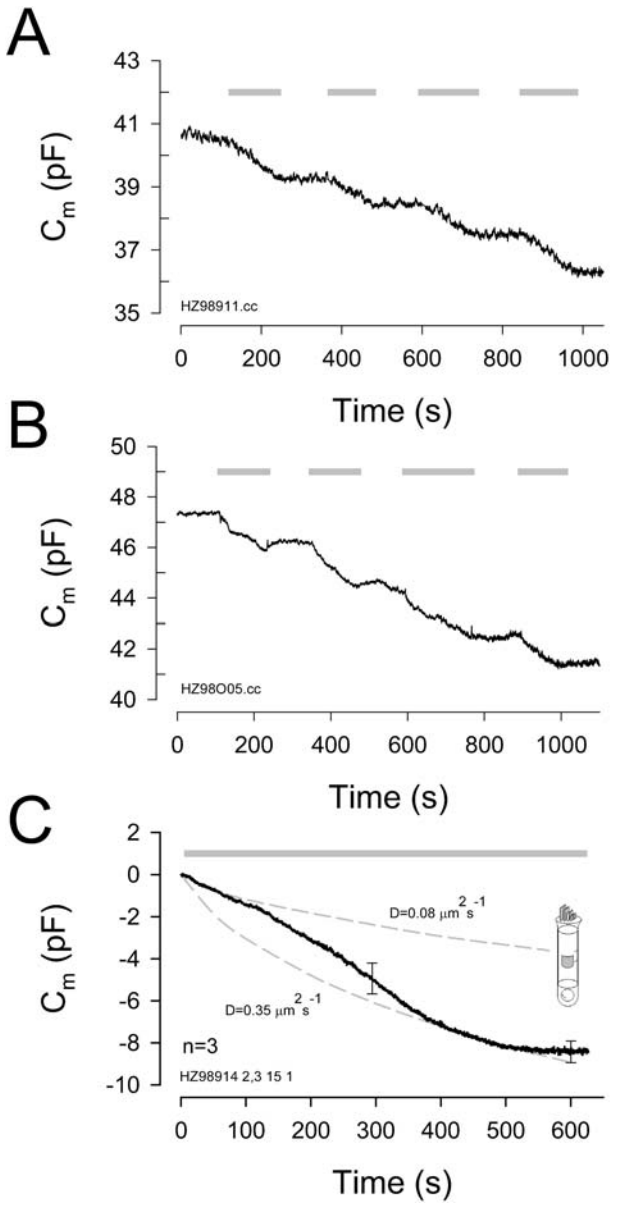

Fig. 3A–C Repeated or continuous exposure of restricted regions of the lateral membrane. **A** OHC filled with Lucifer Yellow showed accumulating decreases in NLC during repeated exposures. The *slope* of decrease appears linear. **B** OHC treated with di-8-ANEPPS showed similar behavior during exposures. **C** Continuous exposure of restricted region (*gray area* in OHC *schematic*) produced an initial linear segment of NLC decrease followed by a decaying rate. The *trace* is the mean±SEM of three OHC responses. The *dotted lines* indicate the result of model simulations with the indicated diffusion coefficients (see Discussion for details)

capacitance or conductance is not likely to be due to diffusion of reactive species far from areas of exposure, since inactivation stops immediately after the light is turned off. This is expected since photo-sensitizers probably act after binding to the membrane, and the estimated lifetime and diffusion of the evolved reactive species, e.g., singlet oxygen, are of the order of a few microseconds, and a hundred nanometers, respectively

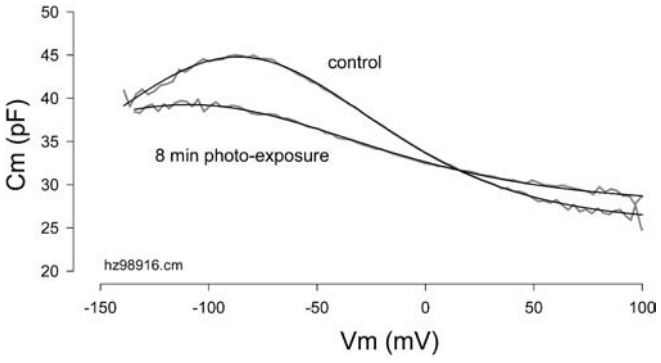

Fig. 4 Functions relating C_m to membrane potential (V_m) before and after an 8-min fiber optic exposure of an OHC treated with di-8-ANEPPS. Fitted parameters: control (V_{pkCm}, z, Q_{max}, C_{lin}, respectively, see text for definitions): −86.5, 0.59, 3.36, 25.5, exposed: −109.4, 0.47, 2.55, 27.7

(see [19] for discussion and references). Thus, the fiber optic exposures that we made probably resulted in highly restricted lesions. Since motors can be inactivated by hydrophilic dyes (Lucifer yellow, 6-carboxyfluorescein, fluorescein-dextran) delivered to the membrane via intracellular or extracellular routes, and by the lipophilic dye (di-8-ANEPPS), susceptible targets are possibly present at intracellular, extracellular and intramembrane domains. The amino acids histidine, methionine, tryptophan and cysteine are most sensitive to reactive oxygen species [13], and these residues are found in all three domains of prestin [32] (J. Zheng, personal communication).

The inactivation of sensitized lateral membrane motors by light may be viewed as a simple irreversible reaction:

$$M^*_+ \xrightarrow[k_{kill}]{h\nu} M_- \qquad (1)$$

For whole cell exposure, where M^*_+ is the sensitized active motor pool, M_- is the inactivated motor pool, and k_{kill} is the inactivation rate constant, the time course of inactivation is a simple exponential, with a time constant of $1/k_{kill}$. For our whole-cell data the rate constant was 0.0384 s^{-1}. If we consider that in this case the non-linear capacitance (NLC) decrease arises from destruction of all motors in the cell (~ 10 million for a low-frequency OHC [10]), we can derive an estimate of motor inactivation rate, namely, about 384,000/s. This high sensitivity to inactivation is mirrored by the inner ear's sensitivity to a variety of insults, including agents that generate reactive oxygen species [6, 16].

The description of the inactivation process in a partially exposed cell is more complicated, as the sensitized active motor pool may be supplied by the unexposed active motor pool, M_+.

$$M_+ \underset{k_{-1}}{\overset{k}{\rightleftharpoons}} M^*_+ \xrightarrow[k_{kill}]{h\nu} M_- \qquad (2)$$

The rate constants k and k-1 will influence the time course of motor inactivation, and actually represent more complex diffusion processes. Thus, the forward rate constant k may be influenced by motor density, motor interactions with other integral membrane proteins and sub-membranous cytoskeleton, and diffusion distances from the irradiated area. The backward rate k-1 will also reflect similar influences, but additionally may suffer from the untoward effects of photo-irradiation, for example, molecular cross-linking.

Peters et al. [18] have performed a conceptually similar study to ours using continuous fluorescence recovery after photobleaching (FRAP) of the dye 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) within a restricted membrane region. They pointed out that in the case where the diffusion time constant (τ_d=irradiated radius^2/D; where D is the diffusion coefficient) is very much greater than the photo-inactivation time constant (τ_k=1/k_{kill}), the time course of fluorescence decay (or in our case capacitance decay) would follow the exponential time course characteristic of photo-inactivation alone. This behavior essentially accounts for the characteristic time of capacitance decrease that we observe under whole-cell photo irradiation. However, in the case where the photo-inactivation process is very much faster than the delivery of motors to the irradiated area, the time course of capacitance decrease would be dominated by the factors that govern the diffusion process. With fiber optic illumination, the time course of capacitance decrease was not a simple exponential (Fig. 3c), as we might have expected for an immobile population of motors, and as we observed with whole-cell irradiation; instead, there was a prolonged linear-like segment followed by an exponentially decaying termination. Furthermore, the magnitude of NLC decrease is far greater than that expected for the estimated exposed area. We conclude from these data that there is, in fact, lateral diffusion of motors into the irradiated area, and that the photo-inactivation process is faster than that of diffusion. This contrasts with observations on Na^+ channels in skeletal muscle that were photo-inactivated under a loose patch pipette; currents did not return for more than 60 min, indicating a diffusion coefficient of less than 0.0001 μm^2/s [26, 29].

The diffusion coefficient of untethered proteins within lipid bilayers typically ranges around 0.01 μm^2/s [7]. That of lipid molecules within the membrane is larger, ranging around 1.0 μm^2/s. The diffusion of the lipid permeable dye di-8-ANEPPS within the OHC lateral membrane is within that range, but interestingly, it is voltage dependent to some extent [14, 15]. This may relate to the voltage dependence of the major protein, prestin, located in that membrane. Another lipid soluble dye, SP-DiIC18, is relatively immobile, but can redistribute within the membrane in a voltage dependent manner and probably depends on prestin activity [30]. In light of these data, it may be possible that the diffusion of prestin itself may depend on the protein's voltage-dependent area state. In the present study, we held the OHCs at a constant membrane voltage, but it will be interesting to evaluate the effects of various holding potentials in future studies.

Given our data, the diffusion coefficient of prestin may be roughly estimated by considering that the problem is basically one of modeling a molecular sink. As a first approximation, we consider an uncapped cylindrical bilayer possessing the surface area (source) of the lateral membrane of a 65-μm-long OHC, 1,288 μm^2, within which reside motors at a density of 7,500/μm^2 (equated to a NLC of 0.01 pF/μm^2) [10]. The motors are considered freely diffusible, but exit the system upon entering a central region (sink) of ~1/20 (81 μm^2) the full surface area. The question is what diffusion coefficient must the molecules possess to produce a decrease in numbers at a rate of 10,000/s (~10 fF/s in the OHC), as we estimate during fiber optic irradiation of the OHC? The problem was modeled in Berkeley Madonna using 1-μm square cells, with a square sink (9 μm edge) in the middle of the OHC (Fig. 3c inset). The dotted lines in Fig. 3c show the time course of reductions in NLC for two diffusion coefficients that bracket the averaged data. A D of 0.08 μm^2/s can account for the initial segment of the data; however, the subsequent segments require larger coefficients. The divergence from a simple and fixed course of diffusion indicates that something more complicated is occurring, perhaps cross-linking of proteins or lipid peroxidation. We must also be aware that the diffusion of the dyes (which themselves may be rendered ineffective in the exposed area) may also influence our measures. We suggest that the earlier segments of the data, where membrane damage may be less, may better represent the diffusion characteristics of prestin and indicate that prestin is untethered. The ability to diffuse may be important for the redistribution of motors after insertion into the plasma membrane during normal motor turnover. Additionally, the absence of tethering may indicate that the proteins freely rotate within the plane of the membrane, and are unlikely to acquire any anisotropic characteristics owing to diffusional constraints.

In sum, we have shown that the OHC membrane motor is susceptible to photo-inactivation, and that this sensitivity can be exploited to reveal the protein's ability to diffuse within the lateral membrane. With the recent molecular identification of the motor [25, 31] we can expect more detailed examinations of the protein's activities in the lateral membrane.

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