Structural Microdomains in the Lateral Plasma Membrane of Cochlear Outer Hair Cells

MING ZHANG AND FEDERICO KALINEC

Section on Cell Structure and Function, Leslie & Susan Gonda Department of Cell and Molecular Biology, House Ear Institute, Los Angeles, CA 90057, USA

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ABSTRACT

The basal and lateral regions of the plasma membrane of cochlear outer hair cells are structurally and functionally distinct. The lateral region contains thousands of motor proteins but few voltage-gated channels. The basal region, conversely, contains a high number of voltage-gated channels but is devoid of motor proteins. It has been suggested that the cortical cytoskeleton is responsible for maintaining this regional distinction. Toward elucidating the structure of the outer hair cell's electromotile mechanism, we investigated the physical organization of the lateral plasma membrane in living guinea pig outer hair cells by analyzing the distribution pattern of the anionic long-chain carbocyanine SP-DiIC₁₈(3) within this area, before and after electrical stimulation and with an intact and a disrupted cytoskeleton. We observed punctate, intensely fluorescent patches as well as areas of weaker fluorescence, with clear local maxima and minima, upon labeling the cells with this membrane-soluble probe. This discrete distribution of SP-DiIC₁₈(3) suggests that the lateral plasma membrane of guinea pig outer hair cells may be composed of small structural domains (microdomains). Disrupting the cytoskeleton with either trypsin or toxin B from *Clostridium difficile* did not change this pattern of distribution, thus indicating that this treatment did not facilitate the lateral diffusion of the probes. Electrical stimulation using whole-cell patch-clamp tech-

niques, on the other hand, induced two responses: fast motility and reversible displacement of the fluorescent probes. Both responses were inhibited by internal perfusion with salicylate, while disruption of the cytoskeleton did not inhibit OHC fast motility but affected the electrically induced redistribution of fluorescent probes. Together, these results suggest that the lateral plasma membrane of guinea pig outer hair cells contains structural microdomains and that the cytoskeleton does not appear to be playing a major role in maintaining the lateral separation of these distinct molecular regions.

Keywords: outer hair cells, anionic carbocyanines, membrane domains, cytoskeleton, guinea pigs, mammalian cochlea

INTRODUCTION

Outer hair cells (OHCs) are sensory motor cells responsible for frequency discrimination and signal amplification in the mammalian inner ear (Dallos 1992; Ashmore and Kolston 1994). OHCs are cylindrical, with a diameter of 8–10 μm and lengths ranging from 30 to 90 μm in guinea pigs. Cell shape is maintained by an elevated internal hydrostatic pressure combined with a two-dimensional actin-spectrin cytoskeleton located just beneath the lateral plasma membrane (Holley et al. 1992; Holley 1996). This cytoskeleton, also known as the “cortical lattice,” is not a continuous structure but a collection of discrete “microdomains” that abut each other at various angles and connect to the plasma membrane through 25 nm long, rodlike structures (pillars) placed about 40 nm apart (Holley et al. 1992).

Current address for Dr. Ming Zhang: Department of Communication Disorders • Texas Tech University Health Sciences Center • Lubbock • TX 79430.

Correspondence to: Federico Kalinec, Ph.D. • Department of Cell and Molecular Biology • House Ear Institute • 2100 West Third Street • Los Angeles, CA 90057 • Telephone: (213) 353-7030; fax: (213) 273-8088; email: fkalinec@hei.org

There is compelling evidence that the OHC plasma membrane contains defined domains. As with other epithelial cells, the structurally and functionally different apical and basolateral regions are separated by a tight-junction belt. In OHCs, however, the basolateral plasma membrane itself is further divided into two structurally and functionally different regions. The lateral region lacks voltage-gated channels but holds thousands of integral membrane proteins ($\sim 7000/\mu\text{m}^2$) (Gulley and Reese 1977; Forge 1991; Kalinec et al. 1992; Huang and Santos-Sacchi 1993; Kalinec and Kachar 1995; Souter et al. 1995; Holley 1996; Santos-Sacchi et al. 1997). These integral membrane proteins are currently associated with the motor molecules responsible for the reversible and very fast changes in cell length known as OHC electromotility (Geleoc et al. 1999; Zheng et al. 2000; Belyantseva et al. 2000; Oliver et al. 2001). The basal region, in contrast, displays a significant number of voltage-gated channels but has few motor molecules (Kalinec et al. 1992; Santos-Sacchi et al. 1997). Since membrane fluidity in guinea pig OHCs seems to be similar to those reported for other eukaryotic membranes (Oghalai et al. 2000), a still unknown mechanism or structure must maintain these different domains. As suggested by Santos-Sacchi and co-workers, the best candidate for this role is the cortical cytoskeleton (Santos-Sacchi et al. 1997; Takahashi and Santos-Sacchi 2001).

Membrane-soluble fluorescent probes are useful molecular tools for analyzing the structure and dynamics of cell membranes. In OHCs, the short-chain cationic carbocyanine DiOC₆ has been used to investigate the structure of the lateral cisternal membrane system (Pollice and Brownell 1993; Forge et al. 1993). It has also been proposed as a marker to identify isolated IHCs and OHCs. The distinct labeling of the subsurface cisternae delineating the OHC outline is very different than the diffuse fluorescence that accumulates in the IHC cytoplasm (Zajic et al. 1993). In addition, Di-8-ANEPPS (a potentiometric probe) and NBD-C₆-ceramide (a marker for the Golgi complex) have been used to evaluate interactions between the components of the lateral wall and lipid mobility in the OHC plasma membrane (Oghalai et al. 1998, 1999, 2000). Long-chain carbocyanines, however, have been used infrequently in the study of OHCs and only as structural controls (Forge et al. 1993). In contrast to short-chain carbocyanines, which interact weakly with the lipidic bilayer, long-chain molecules are excellent markers of the cell plasma membrane with a negligible penetration into the cytoplasm (Haugland 1999). Moreover, since long-chain carbocyanines insert themselves preferentially in rigid, gel-like lipidic domains (Klausner and Wolf 1980; Spink et al. 1990) and are known to

coaggregate with membrane proteins (Thomas et al. 1994), they may be reliable probes to study the structural organization of the OHC lateral plasma membrane.

In the present study we have used the long-chain anionic carbocyanine SP-DiIC₁₈(3) to investigate the structure of the OHC lateral plasma membrane. We found that, in contrast to cationic carbocyanines, SP-DiIC₁₈(3) molecules prefer to insert themselves in highly specific regions of the bilayer. In addition, lateral diffusion is minimal during the time course of our experiments (~ 30 minutes), even after disruption of the cortical cytoskeleton. Electrical induction of OHC fast motility, on the other hand, induced lateral displacement of the fluorescent probes in the OHC plasma membrane. By blocking OHC motility with salicylate and disrupting the cortical cytoskeleton without affecting the motor function with trypsin or toxin B from *Clostridium difficile*, we show that the electrically induced redistribution of fluorescent probes is dependent on cytoskeleton integrity and is not a passive response to membrane potential changes. These results provide evidence of the existence of structural microdomains in the lateral plasma membrane of living guinea pig OHCs and confirm the major role of the cortical cytoskeleton in OHC fast motility. Our results do not support, however, the hypothesis that the cytoskeleton is critical for maintaining the lateral separation of these distinct molecular regions. Preliminary results of a portion of this work have been presented elsewhere (Zhang and Kalinec 2000).

MATERIALS AND METHODS

Cell isolation and labeling

Organs of Corti were obtained from young guinea pigs (200–300 g), euthanized following procedures and protocols approved by the Institutional Animal Care and Use Committee (IACUC). Auditory sensory and supporting cells were dissociated by incubating the organs of Corti (20 minutes at room temperature) in Leibowitz L-15 (Gibco, Gaithersburg, MD) containing 1 mg/mL collagenase (Sigma, St. Louis, MO). Isolated cells were incubated for 20–30 minutes at room temperature with either the anionic sulfo-phenyl dialkyl-indo (DiI) carbocyanine SP-DiIC₁₈(3) at concentrations between 5 and 20 μM or with the short-chain steryl dye Di-8-ANEPPS (75 μM) (both from Molecular Probes, Eugene, OR). Stock solutions of the fluorescent probes were prepared in DMSO following manufacturer protocols. Final concentrations were prepared in phosphate-buffered saline (PBS) at pH 7.4 and used immediately. This report is based on observations of over 100 individual OHCs.

Microscopy and image analysis

Confocal microscopy was performed using an inverted LSM-410 laser confocal microscope (Zeiss, Germany), with a C-apochromat 40X/1.2 water-immersion objective. Drops containing labeled cells in suspension were deposited directly onto glass coverslips allowing access of the patch pipettes. Patched cells were not permitted to attach to the glass because this could interfere with electromotility. Using a pinhole of 20, the thickness of the optical slices in confocal images was estimated to be $1.3\ \mu\text{m}$. This was enough to obtain the image of the dorsal surface of the cell (as opposed to the ventral surface which is closer to the glass coverslip), centered at the cell's longitudinal axis and covering approximately 30% of the total lateral surface of the OHC plasma membrane. Lateral diffusion of SP-DiIC₁₈(3) was investigated by direct examination of small rectangular areas ($<100\ \mu\text{m}^2$) of OHC plasma membrane, photobleached by continuous excitation with the confocal microscope laser beam. To do that, sectors of the plasma membrane of well-stained OHCs were observed at the highest magnification with a 1 s scanning raster for approximately 60 s. This resulted in the bleaching of the sector, with a sharp edge between the bleached and unbleached regions (see Fig. 2). Pairs of images of the photobleached spot and adjacent (unbleached) areas were recorded, the first one immediately after photobleaching and the second one at intervals ranging from seconds to 30 minutes. Ten pairs of images were analyzed in this experiment, with each pair corresponding to a different cell. Images were analyzed offline using Adobe® Photoshop® 4.0 (Adobe Systems, San Jose, CA) and the NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Briefly, profiles of fluorescent intensity were obtained in 100×300 -pixel rectangular areas of the OHC plasma membrane covering photobleached, unbleached and border regions. Identical areas were evaluated in each pair of images, and qualitative and quantitative comparisons were performed between profiles corresponding to every pixel row as well as between the average fluorescence intensity profile along the rectangular area. Fluorescence recovery in the lateral plasma membrane of OHCs labeled with SP-DiIC₁₈(3) was always below or close to the limit of resolution of our system, even after 30 minutes of recovery time. Therefore, diffusion coefficients were not estimated.

Electrical stimulation

For studies involving electrical stimulation, OHCs labeled with SP-DiIC₁₈(3) were placed on the stage of the laser confocal microscope and patch clamped

($-50\ \text{mV}$ holding potential) using an EPC-9 amplifier (HEKA, Pfalz, Germany) and a Patchman electronic micromanipulator (Eppendorf, Hamburg Germany). Pipettes with access resistance between 4 and $5\ \text{M}\Omega$ (corrected at least 80%) and whole-cell currents below $2\ \text{nA}$ were currently used in these experiments. Capacitance compensation was also applied and the voltage-clamp time constant was $<0.5\ \text{ms}$, with an estimated voltage-clamp error $<4\ \text{mV}$. Cells were internally perfused through the patch pipette either with electrode solution (control: $140\ \text{mM}$ KCl, $2\ \text{mM}$ MgCl₂, $0.28\ \text{mM}$ CaCl₂, $0.5\ \text{mM}$ EGTA, and $5\ \text{mM}$ Hepes, adjusted to pH 7.4 with KOH), electrode solution plus salicylate [$10\ \text{mM}$ (Sigma, St. Louis, MO)], trypsin [$150\ \mu\text{g}/\text{mL}$ (Sigma, St. Louis, MO)], or toxin B from *Clostridium difficile* [$10\ \text{ng}/\text{mL}$ (List Laboratories, Campbell, CA)]. Three to five minutes after cell patch, OHC electromotility was elicited by changing membrane potential. Depolarization induced cell shortening and hyperpolarization induced cell elongation. First, cells were depolarized ($+50\ \text{mV}$) and confocal images of the lateral plasma membrane (one per cell) were digitally recorded immediately after depolarization. Then cells were hyperpolarized ($-150\ \text{mV}$) and a second confocal image was recorded. Linear displacements of membrane-soluble fluorescent probes on the plane of the OHC plasma membrane were measured offline in these pairs of images of shortened and elongated (depolarized and hyperpolarized, respectively) cells. Conditions were standardized by positioning the cell images vertically on the computer screen, with the apical region of the cell on top and the patch pipette on the right side. Arbitrary "longitudinal axes" were defined connecting the center of the image of depolarized cells at three different levels with a straight line. Images of bending cells (those where the three points did not remain aligned after hyperpolarization) were not included in the study. Conspicuous, brilliant fluorescent patches were identified on every pair of depolarized-hyperpolarized cell images. We found that the centroid of each patch was not a reliable positional referent because of its dependence on threshold selection. In contrast, the position of the brightest pixels in the patch (indicator of the maximal density of fluorescent probes) was independent of the patch's area and shape as well as of the image threshold. Therefore, the brightest pixel was chosen as a referent of the patch position (target point). The magnitude (d) and direction (α) of the relative displacements of the targets were estimated from the coordinates of every target point (x, y) and a reference point (x_0, y_0) located on the longitudinal axis at the level of the patch pipette attachment site (Kalinec and Kachar 1995). The angle of every displacement vector was measured with respect to the cell's longi-

tudinal axis oriented toward the apex (Kalinec and Kachar 1995). Therefore, a $+90^\circ$ angle means that the displacement vector is perpendicular to the longitudinal axis and oriented to the right, and a -90° angle indicates that the perpendicular vector is oriented to the left. The values of the radial component of the displacement vectors were corrected to eliminate the bias associated with the hyperpolarization-induced decrease in cell diameter.

The area ratios of the same fluorescent patch in depolarization–hyperpolarization image pairs were measured using the functions “threshold” and “area measurement” built into the NIH Image program. Briefly, the function “threshold” segments an image into objects of interest and background on the bases of gray level. When thresholding is enabled, objects are displayed in black and background in white. Because the area values will depend on the grayscale level selected as defining the boundary of our object (threshold level), we performed area comparisons at three different threshold levels chosen to cover the range where the kept objects its original aspect. Statistical analysis was performed by using the log-likelihood ratio (G–) test of independence with the Williams correction (Sokal and Rohlf 1995).

RESULTS

Distribution of SP-DiIC₁₈(3) in the plasma membrane of auditory sensory and supporting cells

SP-DiIC₁₈(3) labeled the plasma membranes of auditory sensory and supporting cells (Fig. 1). No labeling was observed in nuclear, mitochondrial, or other internal cell membranes, suggesting a negligible penetration of the probe into the cytoplasm of any intact cell under our experimental conditions. SP-DiIC₁₈(3) distribution in the lateral plasma membrane of Hensen and other organ of Corti cells was homogeneous. In OHCs, SP-DiIC₁₈(3) distribution was seen as a pattern of bright patches separated by areas displaying a weaker fluorescence with clear local maxima and minima (Fig. 1). At the level of the tight junction belt, labeling was consistently stronger and more uniform than in other regions (Figure 1B, arrowhead). Changes in incubation time as well as in dye concentration did not induce any substantial change in the probe distribution pattern.

Lateral diffusion of SP-DiIC₁₈(3) molecules in the OHC plasma membrane was extremely slow. Rectangular areas of OHC plasma membrane, photobleached by continuous excitation with the confocal microscope laser beam, did not show significant changes in fluorescent intensity, even after 30 minutes of recovery time (Fig. 2 A,B). This was confirmed

FIG. 1. SP-DiIC₁₈(3) labeling of the plasma membrane of (A, B) OHCs and (C, D) Hensen cells. Midcoronal optical sections (A, C) show that only the plasma membranes were stained, with negligible penetration of the dye into the cytoplasm. The pattern of labeling in OHCs is punctuated (A, arrows), suggesting a nonuniform incorporation of this carbocyanine into the OHC lateral plasma membrane. B and D show the cells optically sectioned at the level of the plasma membrane. The patchy signal observed in the OHC plasma membrane (B) is different with respect to the uniform distribution of the probe in the Hensen cell (D). A, B. Remnants of nerve terminals' membranes at the OHC basal pole are intensely fluorescent. Scale bars = 2 μ m.

by comparing the profiles of fluorescent intensities at different times (Fig. 2C). Diffusion coefficients were not estimated because fluorescence recovery was below the limit of resolution of our system. These results suggest that SP-DiIC₁₈(3) molecules become essentially immobile when partitioned into the OHC lateral plasma membrane. As a control, we labeled OHCs with Di-8-ANEPPS, a cationic styryl dye used to measure lipid mobility in the plasma membrane of guinea pig OHCs (Oghalai et al. 1999, 2000). In agreement with reported results, Di-8-ANEPPS probes distribute homogeneously and show a fast lateral

FIG. 2. Lateral diffusion of SP-DiIC₁₈(3) in the OHC plasma membrane. Regions of SP-DiIC₁₈(3)-labeled OHCs were selectively bleached by continuous excitation with the confocal microscope laser beam. Comparisons between images recorded immediately after bleaching (**A**) and up to 30 minutes later (**B**) show no significant differences in the distribution of the fluorescent probes. **C.** Densi-

tometric profiles of fluorescent intensity from the small rectangular regions indicated in **A** and **B**. Note the lack of significant diffusion of fluorescent probes into the photobleached region, suggesting that fluorescent probes are essentially immobilized in the OHC plasma membrane.

diffusion in the plasma membrane of OHCs as well as in other cell populations present in our samples (results not shown).

We speculated that the confinement of the anionic carbocyanines to distinct regions of the OHC lateral plasma membrane could be due to the presence of cytoskeletal constraints. To test this, we used the ability of intracellular trypsin perfusion to completely disrupt the cytoskeleton and the endomembranous system of OHCs (Kalinec et al. 1992; Huang and Santos-Sacchi 1994; Kakehata and Santos-Sacchi 1996; Adachi and Iwasa 1999). SP-DiIC₁₈(3)-labeled OHCs were internally perfused with trypsin (150 μ g/mL). Pairs of confocal images were taken at the start of the perfusion and 20 minutes later. We observed that disruption of the cortical cytoskeleton led OHCs to lose their characteristic cylindrical shape, but it did not significantly change the general pattern of distribution of SP-DiIC₁₈(3) in the plasma membrane (Fig. 3A–D). Unfortunately, trypsin-perfused cells were very

unstable, and photobleaching experiments aimed at detecting small changes in lateral diffusion of the membrane probes did not provide reliable results. Exposure of trypsin-perfused cells to the laser beam accelerated the cell's rounding process, with consequent problems in maintaining a stable focal plane, and frequently resulted in the disruption of the cell membrane. Similar problems were observed in OHCs internally perfused with toxin B from *Clostridium difficile*, a cytotoxic agent with the ability to depolymerize actin filaments (Just et al. 1995; Steele-Mortimer et al. 2000).

Changes in distribution of SP-DiIC₁₈(3) in the plasma membrane of electrically stimulated OHCs

Labeled cells were patch clamped and successively depolarized and hyperpolarized, with the consequent shortening and elongation of the cells. Although the general appearance of the membrane was similar

FIG. 3. Distribution of SP-DilC₁₈(3) in the plasma membrane of trypsin-perfused OHCs. **A, B.** Optical sections of an OHC before internal perfusion with trypsin. **C, D.** The same cell after 15 minutes of trypsin digestion. The cell became rounded, limiting the size of the plasma membrane surface in the focal plane of the confocal microscope, but the discrete distribution of SP-DilC₁₈(3) is still clearly visible.

under depolarizing and hyperpolarizing conditions (Fig. 4A,B), closer examination of the images showed redistribution of the fluorescent probes, with changes in number and position of the local minima and maxima of the distribution (Fig. 4C,D). We measured the area ratios ($A_{\text{hyp}}/A_{\text{dep}}$) of 20 different fluorescent patches in depolarization–hyperpolarization image pairs at three different threshold values. The averages (expressed as mean $\pm$ SEM) were 1.40 ± 0.07 , 1.43 ± 0.06 , and 1.64 ± 0.09 . These results suggest that the area occupied by the fluorescent dyes consistently increased in response to cell hyperpolarization. Interestingly, in comparison to the depolarized state, a slight increase in fluorescent intensity was consistently observed in hyperpolarized OHCs. This phenomenon was not observed in Hensen cells electrically stimulated in a similar way.

FIG. 4. Optical section at the level of the plasma membrane of an electrically stimulated OHC stained with SP-DilC₁₈(3). **A.** Depolarized cell. **B.** Hyperpolarized cell. **A.** The arrowhead indicates the patch pipette. Scale bar = 2 μm . **C, D.** High magnification of the region enclosed in the rectangular area displayed in **A** and **B**, respectively. Note the changes in distribution of the lipid-soluble probe as well as the general increase in the fluorescence of the area induced by membrane hyperpolarization. Optical noise was partially removed by digital filtering ("minimum" filter in Adobe Photoshop). Scale bar = 1 μm .

To test reversibility of the electrically induced redistribution of the fluorescent probes, we compared pairs of confocal images of the same cells acquired, respectively, before and after a full depolarization–hyperpolarization cycle. Following the cycle, the brilliant fluorescent patches reverted to shapes and sizes similar, but not identical, to those seen in the cells prior to the cycle (Fig. 5). We calculated the area ratio for 30 different patches and obtained a mean value of 0.97 ± 0.02 . This ratio indicates that the area of the patch after a depolarization–hyperpolarization cycle is slightly smaller than before the cycle. However, this difference might be attributable to photobleaching of the sample. The pattern of distribution of the weaker fluorescence, which changes significantly with hyperpolarization (Fig. 4C,D), reverts only partially when the cell is returned to the depolarized

FIG. 5. Electrically induced displacement of the SP-DiIC₁₈(3) probes in OHC plasma membrane is not fully reversible. **A.** Distribution of SP-DiIC₁₈(3) in the plasma membrane of a depolarized OHC. **B.** The same cell after being hyperpolarized for a 15-s period and depolarized again. Note that both images are virtually identical. **C,D.** Magnification of the small rectangular areas indicated in **A** and **B**, respectively. Note changes in the distribution of the probes that cannot be associated with photobleaching. Optical noise was partially removed by digital filtering ("minimum" filter in Adobe Photoshop).

state. Although we did not evaluate changes in size and shape, differences were clearly detected (compare Fig. 5C with 5D).

Displacement of the fluorescent probes is driven by the OHC electromotile mechanism

Lateral displacement of the anionic carbocyanines in electrically stimulated OHCs could be a passive response to the electrical changes or a consequence of the activation of the membrane-embedded electromotile mechanism. Therefore, we investigated how the lateral displacement of the fluorescent probes was affected by the disruption of the two main components of the OHC electromotile mechanism: the motor molecules and the cortical cytoskeleton.

Internal perfusion of OHCs with salicylate (10 mM), which is thought to inhibit up to 90% of OHC electromotility by a direct action on the molecular motors or their lipidic environment (Tunstall et al. 1995; Kakehata and Santos-Sacchi 1996), also inhibited the displacement of membrane dyes. With the exception of a few cases, the magnitude of the dis-

placement vectors was below the resolution of our measurement system (results not shown). The pattern of distribution of fluorescent probes in the plasma membrane of salicylate-treated cells was similar to that observed in nontreated cells. In contrast, we did not observe significant changes in fluorescence intensity in response to hyperpolarization in salicylate-treated OHCs.

To investigate the role of the cortical cytoskeleton on the lateral displacement of SP-DiIC₁₈(3), we measured the displacement vectors (moduli and angles) of fluorescent spots in patch-clamped OHCs internally perfused with either trypsin or toxin B from *Clostridium difficile* (Fig. 6). Trypsin treatment resulted in the near complete digestion of the OHC cortical cytoskeleton within a few minutes, but it did not inhibit the action of the membrane-embedded motor molecules (Kalinec et al. 1992; Huang and Santos-Sacchi 1994; Kakehata and Santos-Sacchi 1996). Toxin B, in turn, is a substance that disturbs the actin cytoskeleton through the inhibition of the small GTPases RhoA, Rac1, and Cdc42 (Just et al. 1995; Steele-Mortimer et al. 2000). As a control condition for these experiments, we measured displacement vectors on images of depolarized and hyperpolarized cells perfused with electrode solution.

Displacements of up to 2.4 μm were observed under the control condition, with a positive correlation ($r = 0.77$) between their magnitudes and the distance of the fluorescent domains to the pipette attachment site (Fig. 6A). These values are consistent with previously reported measurements of the displacement of intracellular particles (Holley and Ashmore 1988) and carboxylate microspheres attached to the plasma membrane of electrically stimulated OHCs (Kalinec and Kachar 1995). Moreover, displacements were reversible. After a full depolarization-hyperpolarization cycle, the fluorescent patches return to their original positions, suggesting that their displacement was driven by the membrane-embedded molecular motors rather than by facilitated diffusion. Figure 6B shows the orientation of the displacement vectors as a frequency distribution in 10° angle classes. The full range was around 180°, with 20% of the vectors having angles $\pm 40^\circ$. The estimated mean orientation was -0.71° nearby parallel to the longitudinal axis of the cell, with the mode in the 0° to -10° class. If molecular motors drive membrane probe displacement, then these results suggest that the motors are organized in microdomains with a predominant direction parallel to the cell longitudinal axis.

To be consistent with the control conditions, measurements on trypsin- and toxin B-perfused OHCs were performed in cells that had maintained their cylindrical shape, indicating only a partial disruption of the cytoskeleton. In contrast to the

FIG. 6. Magnitude and orientation of the electrically induced displacement of fluorescent patches in the OHC plasma membrane. **A.** Displacement of the fluorescent domains over their distance to the pipette attachment site in control cells ($n = 116$). Displacement increases with the distance, with a correlation coefficient $r = 0.77$. **B.** Frequency distribution, in 10° angle classes, of the direction of the displacement vectors with respect to the cell longitudinal axis in control cells. The estimated mean was -0.7° and the mode was in the 0° to -10° class. However, the full range of orientations covered 180° , with 23% of them larger than $\pm 30^\circ$. **C.** In trypsin-perfused cells ($n = 64$), displacement also increases with the distance to the pipette attachment site ($r = 0.77$). **D.** Orientation of the displacement vectors in trypsin-perfused cells was mainly parallel to the major cellular

axis. The mean was 4.6° and the mode was in the 0° to $+10^\circ$ class, with 60 out of 64 displacement vectors oriented in the $\pm 30^\circ$ range. This distribution was significantly different ($p \leq 0.01$) from that of control cells (**B**). **E.** In toxin B-perfused cells ($n = 68$), the correlation between the magnitude of the displacement vector and the distance to the pipette attachment site was slightly lower than those observed in control and trypsin-perfused OHCs ($r = 0.64$). **F.** Displacement vector orientation, in contrast, was similar to that in trypsin-perfused cells but significantly different from that observed in control cells ($p \leq 0.01$; compare **B**, **D**, and **F**). The mean was 0.2° and the mode was in the 0° to $+10^\circ$ class, with 63 out of 68 displacement vectors with angles smaller than $\pm 30^\circ$.

observations in control cells, the displacement vectors in trypsin-perfused cells displayed a relatively constant orientation, with 80% of the probes now moving mainly in a direction roughly parallel to the OHC longitudinal axis ($\pm 30^\circ$, Fig. 6D). Toxin B-treated cells displayed a similar response to trypsin-treated cells (Fig. 6E,F). For instance, the orientation of the displacement vectors was also approximately parallel to the major cell axis, with more than 90% of the values in the $\pm 30^\circ$ range (Fig. 6F). We performed a statistical comparison of the distribution of displacement vector orientations in control, trypsin-, and toxin B-perfused cells by using the log-likelihood ratio (G-) test of independence with the Williams correction (Sokal and Rohlf 1995). While no significant differences were found between trypsin- and toxin B-perfused cells (Fig. 6D,6F, respectively), both distributions were different from the control one (Fig. 6B).

Interestingly, in neither the trypsin- or toxin B-perfused cells did the distribution pattern of SP-DiIC₁₈(3) in the regions between patches change upon OHCs' elongation or shortening (Fig. 7, compare with Fig. 4). The relative position of maxima and minima as well as the general appearance of the distribution were preserved, indicating that the fluorescent probes moved roughly in the same direction. No voltage-induced changes in fluorescence intensity were observed in these cells either. We also measured the area ratios ($A_{\text{hyp}}/A_{\text{dep}}$) of 20 different brilliant fluorescent patches in depolarization-hyperpolarization image pairs at three different threshold values in trypsin-perfused cells. The averages at every threshold level (expressed as mean $\pm$ SEM) were 1.0 ± 0.5 , 1.1 ± 0.6 and 1.1 ± 0.8 . Our observations suggest that the area occupied by the fluorescent dyes in trypsin-perfused cells, in contrast to that in control cells, did not increase in response to membrane hyperpolarization. These results strongly support the hypothesis that the lateral displacement of the anionic probes in the plane of the OHC membrane is not a passive response to electrical stimulation. They also confirm the role of the cortical cytoskeleton as the provider of the vectorial component to the forces generated in the OHC lateral plasma membrane.

DISCUSSION

Overview

The existence of a long-range lateral order in biological membranes, with lipids and proteins segregated in domains whose composition, organization, and function differ from the rest of the membrane, was proposed in the late 1970s by Jain and coworkers (Jain and White 1977; Jain and Wagner 1980). Since

FIG. 7. Cytoskeletal disruption changes the pattern of the electrically induced lateral displacement of SP-DiIC₁₈(3) probes in the OHC plasma membrane. An OHC labeled with SP-DiIC₁₈(3) was microinjected with 150 $\mu\text{g}/\text{mL}$ trypsin through the patch pipette and, after 10 minutes of enzymatic digestion, successively depolarized (A) and hyperpolarized (B). A reticule of thin lines was superimposed onto the images to show that, except for one of the patches (arrow) that clearly moved laterally, the displacement of the membrane probes is nearly parallel to the cell longitudinal axis. C, D. magnified images of the region enclosed by highlighted rectangles in A and B, respectively. Note that membrane hyperpolarization did not change significantly the distribution of fluorescent probes, in contrast to the response observed in control cells (compare with Fig. 4C,D). C, D. Optical noise was partially removed by digital filtering ("minimum" filter in Adobe Photoshop).

then, considerable experimental evidence has accumulated supporting the existence in cell membranes of distinct structural domains from tens of nanometers to a few microns in size. It has been suggested that such domains may result from segregation and self-association of membrane lipids or proteins, or by "corralling" of membrane proteins by cytoskeletal structures acting as fences to prevent their lateral diffusion (Edidin 1993; Kusumi and Sako 1996). This latter mechanism is known as "cytoskeletal fencing" (Kusumi and Sako 1996).

In OHCs, a two-dimensional actin-spectrin cytoskeleton lies a few nanometers beneath the plasma membrane. Moreover, both structures are coupled by thousands of evenly distributed structural pillars, presumably anchoring a similar number of membrane proteins to the cytoskeleton (Raphael and Wroblewski 1986; Flock et al. 1986; Holley et al. 1992;

Souter et al. 1995). Thus, the pillars, together with the molecular organization of the cortical cytoskeleton, may be the defining "corrals" able to prevent lateral diffusion of transmembrane proteins and associated molecules (Edidin 1993; Kusumi and Sako 1996). Our results, however, suggest that molecular self-association—and not cytoskeletal fencing—may be the major mechanism keeping protein segregated in the OHC lateral plasma membrane.

Distribution of SP-DiIC₁₈(3) in the lateral wall of OHCs

Critical to the interpretation of our data is the assumption that SP-DiIC₁₈(3) molecules insert themselves into the OHC plasma membrane. Of course, a hydrophobic ionic dye will have some equilibrium distribution between the external aqueous medium and the cell plasma membrane. There is general agreement, however, that the hydrophobicity of their long, saturated fatty acid chains will drive a very high percentage of these carbocyanine probes to insert deeply into the outer leaflet of the lipidic bilayer (Klausner and Wolf 1980; Spink et al. 1990). The negatively charged sulfonated groups, on the other hand, should interact with the charges in the membrane surface, reducing the tendency of the probes to self-aggregate (Haugland 1999). Unfortunately, the level of resolution offered by light microscopy is insufficient to visualize such subcellular events. We have considered examining both the distribution of the probes and their interaction with the molecular components of the OHC plasma membrane by high-resolution electron microscopy. However, this examination has not yet been possible.

It must be noted that SP-DiIC₁₈(3) probes must first overcome the "electrical barrier" of the predominantly negatively charged cell glycocalyx in order to reach the plasma membrane. This is particularly true in OHCs, where studies with fluorescein-labeled lectins have shown a high concentration of negatively charged glycoconjugates (Gil-Loyzaga and Brownell 1988; Plinkert et al. 1992). Interaction of the probes with the glycocalyx may have at least two direct consequences. First, anionic carbocyanines will incorporate less well and at a slower rate than cationic probes. Second, charge repelling must result in the membrane-incorporated anionic probes inserting even more deeply into the lipidic bilayer. This is an important distinction between anionic and cationic probes, especially those of the latter class that have short hydrophobic chains. Cationic probes would be easily incorporated into the plasma membrane but will be kept closer to the surface of the bilayer by attractive electrostatic interactions with the negative charges of the cell glycocalyx. Thus, it stands to rea-

son that they would be freer to diffuse laterally than anionic, long-chain probes that are deeply inserted into the bilayer.

Analysis of the distribution of SP-DiIC₁₈(3) probes in the OHC plasma membrane shows two clearly differentiated patterns: discrete, bright fluorescent patches and a more extended but weaker fluorescence with clear local maxima and minima. The bright patches may represent accumulations of fluorescent probes. Such a high fluorescent signal may also explain the apparent increase in membrane thickness that is observed. The patches were consistently seen in OHCs at a wide range of dye concentrations. They were not, however, seen in any other cells of the organ of Corti. Moreover, we are not aware of any reports describing similar staining patterns with SP-Dil in other cell types. We suspect these bright patches may be associated with regions of the OHC glycocalyx with a predominance of positively charged or neutral sugar residues. Lack of an effective "electrical barrier" should facilitate the incorporation of the probes into small regions of the plasma membrane, where their lateral diffusion would be prevented later by environmental constraints. In support of this idea, we found that electrically induced changes in the patch area were linked to the integrity of the cytoskeleton, thus implying a direct interaction of the fluorescent probes with cellular components. In addition, the bright patches were practically absent in OHCs that were partially deglycosylated by using the GLYCOPROTM deglycosylation kit (ProZyme, San Leandro, CA), or by cell incubation in L-15 plus trypsin (300 μ g/mL) for 30 minutes (results not shown). In contrast, the more extended pattern of distribution of SP-DiIC₁₈(3) was not affected by partial removal of the glycocalyx, and all the evidence indicates that it corresponds to fluorescent probes that were deeply inserted into the OHC plasma membrane. Altogether, our results suggest that the structure and composition of the lateral plasma membrane of living OHCs is different from the cell membrane of other cell types. This observation is consistent with the unique features of the OHC's lateral plasma membrane, which is thought to be associated with the electromotile mechanism (Gulley and Reese 1977; Forge 1991; Kalinec et al. 1992).

The very slow lateral diffusion of SP-DiIC₁₈(3) contrasts with the fast mobility of the styryl fluorescent probe Di-8-ANEPPS in the OHC plasma membrane. Using Di-8-ANEPPS probes, for instance, Oghalai and coworkers (1999, 2000) reported an average lateral diffusion coefficient of about 4×10^{-9} cm²/s, typical for a very fluid environment. The simplest explanation for this difference is that the short-chained Di-8-ANEPPS probes insert them-

selves in fluid areas of the bilayer while the long-chained SP-DiIC₁₈(3) prefer more rigid, gel-like regions. This explanation, however, raises an intriguing question: Why are the distributions of these two probes in the OHC plasma membrane not complementary? In contrast to SP-DiIC₁₈(3), Di-8-ANEPPS displays a very homogeneous pattern without any indication of the presence of nonfluid areas in the OHC membrane. This is curious because the OHC lateral plasma membrane has about 7000 molecular motors μm^2 (Huang and Santos-Sacchi 1993; Adachi and Iwasa 1999), in addition to other proteins and the lipid molecules tightly associated with them (presumably in gel phase). This particular composition of the OHC plasma membrane suggests that a high percentage of the membrane surface should be composed of nonfluid regions. As an alternate hypothesis, we speculate that Di-8-ANEPPS molecules may distribute preferentially in the external surface of the outer leaflet of the bilayer, with only a weak interaction with the clusters of motor proteins and, consequently, few restrictions to lateral diffusion. SP-DiIC₁₈(3) probes, in contrast, would be inserted deeply in well-defined regions of the OHC plasma membrane and their lateral diffusion could be prevented either by the low fluidity of those regions, local constraints, or because of interactions of the fluorescent probes with specific components of the plasma membrane. While the distribution of Di-8-ANEPPS is quite homogeneous, the presence of local maxima and minima in the distribution of SP-DiIC₁₈(3) supports the existence in the OHC plasma membrane of small regions with different levels of accessibility for these membrane probes. This provides strong evidence of the existence of small structural domains (in the micrometer range) in the lateral plasma membrane of living guinea pig OHCs. Interestingly, the existence of functional microdomains in the OHC lateral wall has been suggested recently by electrophysiological measurements (Santos-Sacchi et al. 2000; Takahashi and Santos-Sacchi 2001).

Role of the cortical cytoskeleton

Because of the small size of SP-DiIC₁₈(3) molecules, the cortical cytoskeleton cannot directly prevent their lateral diffusion in the plane of the OHC lateral plasma membrane. It may, however, establish local constraints to the diffusion of integral membrane proteins, facilitating the aggregation of membrane components in well-defined structural microdomains. It has been proposed that the restriction to the lateral diffusion of proteins provided by the cytoskeleton is far greater than that provided by the molecular structure of the membrane itself (Kusumi and Sako

1996). Therefore, it can be predicted that even a partial removal of these cytoskeletal constraints should result in an enhanced redistribution of membrane components, including membrane-soluble fluorescent probes. The results reported here, however, failed to show significant changes in the lateral diffusion of SP-DiIC₁₈(3) induced by disruption of the cortical cytoskeleton with trypsin or toxin B from *Clostridium difficile*. Our results are further supported by recently reported experiments on two OHCs internally perfused with pronase, a wide-spectrum proteolytic enzyme (Takahashi and Santos-Sacchi 2001). By measuring gating currents before and after the pronase-induced cytoskeletal disruption, these authors did not detect lateral diffusion of molecular motors into the basal pole of the cells. Therefore, we can conclude that the molecular structure of the membrane itself, rather than cytoskeletal fencing, is the major mechanism for maintaining molecular segregation in the OHC basolateral plasma membrane. It cannot be ruled out, however, that the cytoskeleton was involved in the generation of this membrane structure, facilitating the aggregation of membrane molecules and, perhaps, as part of the membrane-sorting mechanism that targeted those molecules to the OHC lateral plasma membrane.

Lateral displacement of the fluorescent probes is driven by the OHC electromotile mechanism

Although anionic carbocyanines seem to be essentially immobilized in the lateral plasma membrane of living cochlear OHCs, we have shown that changes in membrane potential induced their redistribution in the plane of the membrane. Since this redistribution was inhibited by salicylate, trypsin, and toxin B, we concluded that the OHC electromechanical transduction mechanism drives it instead of being a passive response to electrical changes. Moreover, since salicylate affects the function of the motor proteins and trypsin and toxin B disrupt the cytoskeleton, these results suggest that both components of the electromotile mechanism are required for the voltage-induced response.

The previous statement leads to an intriguing question. How can disruption of the cytoskeleton inhibit redistribution of SP-DiIC₁₈(3) if the motors are still functional? We envisioned a possible scenario. First, the fluorescent probes might be inserted into regions of the OHC plasma membrane that are relatively free of motor proteins (there are no motor proteins "between" fluorescent probes). Second, probes might be inserted into regions of low lateral compressibility (possibly in gel phase which is preferred by long-chain carbocyanines), surrounded by

microdomains rich in motor proteins. In trypsin-treated cells, the motors would generate the changes in cell length, but the fluorescent regions would move back and forth without changes in area or in the pattern of distribution of the probes (as shown in Fig. 7). With an intact cytoskeleton providing a vectorial component to the forces generated by the motors, in contrast the fluorescent regions would be distorted by the shearing stresses generated at their boundaries, with a consequent change in the general distribution of the probes (as shown in Fig. 4C,D).

The proposed scenario is also consistent with the voltage-induced increase in fluorescent intensity observed in OHCs but not in Hensen cells, nor in OHCs treated with salicylate, trypsin, or toxin B. The more plausible explanation to this response involves "fluorescence quenching," a decrease in fluorescence intensity due to aggregation of the probes, which has already been described for carbocyanines (Loew 1988; Haugland 1999). Because of fluorescence quenching, the fluorescence intensity in depolarized OHCs would be lower than expected. Upon hyperpolarization, however, changes in the distribution of the dye in the membrane would decrease the quenching effect resulting in a consequent increase in fluorescence intensity. Therefore, the lack of voltage-induced changes in fluorescence intensity in Hensen cells or OHCs treated with salicylate, trypsin, or toxin B could be associated with the lack of redistribution of the probes under these experimental conditions.

CONCLUSION

We have investigated the distribution of the long-chain anionic carbocyanine SP-DiIC₁₈(3) in the plasma membrane of living guinea pig OHCs. We found that this fluorescent probe was incorporated in well-defined domains in this membrane and did not diffuse laterally in the plane of the membrane during the time course of our experiments, even after disruption of the cortical cytoskeleton. These results provide the first evidence of the existence of structural microdomains in the plasma membrane of living OHCs and suggest that cytoskeletal fencing is not a major mechanism for maintaining molecular segregation in the OHC lateral plasma membrane.

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