DISTRIBUTION OF ANIONIC SITES ON THE OVIDUCT CILIARY MEMBRANE

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ABSTRACT

Polycationic ferritin (PCF) was used as a visual probe for anionic sites on the oviduct ciliary membrane. The binding of PCF to ciliary membranes was dependent on the concentration of the probe in the incubation media. At low concentrations (0.08-0.16 mg/ml), PCF was bound exclusively to the tip of the cilium whereas at higher concentrations (0.32-0.64 mg/ml), ferritin was located at the tip and at the base around the transition region, with occasional scattered clumps on the remainder of the membrane. The base and tip binding was found to be associated with special surface modifications of the membrane in these regions. At the tip, PCF was bound to a filamentous glycoealyx termed the ciliary crown. Base binding was associated with a system of five to six 140-Å high ridges, each of which encircled the membrane of the transition region. The ridges were equally spaced (~245 Å spacing) along the length of the transition region. Since pretreatment of oviduct with either neuraminidase or protease blocked the binding of the probe, the PCF-binding sites appear to be negatively charged glycoproteins or mucopolysaccharides.

The ciliary membrane is an important component of the cilium that is involved both in the regulation of ionic and metabolic states within the axoneme (7, 22) and in determining specific interactions with its environment (24, 25). Electron microscope studies show that this membrane sometimes has special surface modifications, e.g., hair-like appendages on Chlamydomonas flagella called mastigonemes (16). In addition, most cilia have two to six rows of intramembrane particles that encircle the basal portion of the membrane. These particles, termed the ciliary necklace (11, 21, 23, 28), are thought to be involved in the regulation of ion transport (11). The remainder of the membrane usually has randomly distributed intramembrane particles; however, in some cases, there are rows of particles that run the longitudinal length of the cilium (23), or short longitudinally oriented rows of particles (the ciliary plaque [20, 23]) that are located just above the ciliary necklace.

The oviduct ciliary membrane has a ciliary necklace consisting of six rows of particles (11), randomly arranged intramembrane particles in the rest of the membrane, and a surface modification at the tip of the cilium called the ciliary crown (5). In a longitudinal view, this crown structure appears to be composed of a tuft of 225-Å-long fibers covering the surface of the tip; however, in cross section, it appears to be a system of ridges that cover the membrane. The ciliary crown has also been found on the tips of thymic cyst cilia in “nude” mice (3), and a similar surface modification is found on the tips of oral cilia in Tetrahymena pyriformis (23).
The presence of surface modifications and intramembrane particles as part of the oviduct ciliary membrane suggests that there might be cell surface determinants such as lectin-binding sites, anionic sites, or other cell surface receptors that are important for ciliary function. We believe that the anionic site (site of negative charge) might be of particular importance in oviduct cilia because of the role that this organelle plays in gamete transport (1, 15). That is, charge interactions between the cilium and the gamete may take place during transport.

The localization of anionic sites on surface membranes has been of interest because of the large net negative charge that exists on the surfaces of many cells (18, 26) and because these charges may be involved in specific cell-cell and cell-environment interactions (26). The distribution of negative sites on the cell surface has been studied with the use of colloidal iron as the visual marker (2, 10, 17, 19, 27, 29, 30). However, more recently, Danon et al. (4) introduced the use of polycationic ferritin (PCF) for localizing anionic sites. This electron-dense probe is now preferred because it is possible to treat live cells at a neutral pH, thus avoiding the introduction of fixation (12) and pH artifacts (10, 27).

In the present study, the distribution of anionic sites on the surface of the oviduct ciliary membrane was examined with the use of PCF. Our goal was to correlate the binding of PCF with known aspects of the ciliary membrane structure and to compare the distribution and behavior of anionic sites on this membrane with those of other membranes that have been studied.

MATERIALS AND METHODS

All studies were carried out on 5-kg New Zealand white rabbits. Animals were sacrificed by an intravenous injection of sodium pentobarbital (Abbott Laboratories, Chicago, Ill.). Oviducts were removed and placed in Hanks' balanced salt solution (HBSS). After the removal of excess fat, the oviduct was cut open to expose the epithelial surface. For each treatment, pieces were cut from the fimbriated end of the oviduct.

Treatment with PCF

PCF (Miles Laboratories, Inc., Elkhart, Ind.) was diluted to concentrations ranging from 0.08 to 0.64 mg/ml with 0.1 M Na phosphate buffer, pH 7.3. Pieces of excised oviduct were washed in phosphate buffer and then incubated at 25°C for various periods of time in PCF. After the incubation, the pieces of tissue were washed in phosphate buffer and fixed in either: (a) 2% glutaraldehyde in 0.1 M Na phosphate buffer, pH 7.3; (b) 1% glutaraldehyde, 1% tannic acid in 0.1 M Na phosphate buffer, pH 7.3; or (c) 2% glutaraldehyde, 6% paraformaldehyde, 0.02 M collidine in 0.02 M N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid buffer, pH 7.3. The glutaraldehyde-tannic acid fixative gave the best membrane preservation; however, it did change the appearance of the PCF. Regardless of the fixative, the distribution of PCF binding was the same. The same procedure was used with pretreated tissue, untreated tissue, and with experiments to test the binding of native ferritin (Polysciences, Inc., Warrington, Pa.). In the latter case, tissues were exposed to 0.64 mg/ml native ferritin for 10 min.

Enzyme Pretreatment of Tissue

To characterize PCF-binding sites, we tested the effect of proteases and neuraminidase on PCF binding. Pieces of oviduct were incubated in the presence of the following enzymes: (a) 1 and 2 mg/ml pronase (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.1 M Na phosphate buffer, pH 7.3, and then exposed to PCF (0.32 mg/ml) for 5 min at 25°C. No alteration in the ciliary beat could be detected after treatment with these enzymes.

PCF Binding to Nonmotile Cilia

Two techniques were used to stop the ciliary beat. In some cases, pieces of oviduct were fixed for 10 min in 1% paraformaldehyde buffered with 1% sodium cacodylate pH 7.4, washed in phosphate buffer, and then exposed to 0.32 mg/ml of PCF for 60 min. In other trials, tissue was incubated in phosphate-buffered 100 /&M dinitrophenol (Sigma Chemical Co.) until the cilia stopped beating (~35 min). The tissue was then incubated in a 0.32-mg/ml solution of PCF that contained 100 /&M dinitrophenol in 0.1 M Na phosphate pH 7.3 for 15 min. As a control, tissue was incubated directly in the PCF-dinitrophenol mixture.

Pretreatment with Poly-L-Lysine

After a wash in 0.1 M Na phosphate buffer, pH 7.3, pieces of oviduct were exposed to poly-L-lysine (Sigma Chemical Co.). Three different ranges of molecular weights were tested: (a) mol wt >70,000 at 0.1 mg/ml in 0.1 M Na phosphate, pH 7.3, for 10 min; (b) mol wt 3,400 at 0.3 mg/ml in 0.1 M Na phosphate, pH 7.3, for 10 min; (c) mol wt 15,000 at 0.3 mg/ml in 0.1 M Na phosphate, pH 7.3, for 10 min. That is, charge interactions between the cilium and the gamete may take place during transport.
phosphate, pH 7.3, for 10 min. After treatment, the tissue was washed and then incubated in 0.32 mg/ml PCF for 5 min.

Electron Microscopy

Fixed pieces of tissue were postfixed in 1% sodium cacodylate-buffered 1% OsO₄, pH 7.3, and embedded in Araldite. Sections were cut on a DuPont-Sorvall MT-2B ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.) and examined with a Philips 200 electron microscope.

RESULTS

The epithelial surface of the oviduct is composed of equal portions of ciliated and secretory cells. In addition, the epithelium covers a highly convoluted and folded stroma; therefore, not every portion of the epithelial surface is directly exposed to agents in the medium. This factor had to be considered in evaluating the binding of PCF to ciliary membranes. It proved to be of value, however, that for a given region both ciliated and secretory cell membranes were equally exposed to any experimental treatment.

The ciliary membrane can be divided into three regions; the tip region (Fig. 1), the main region (Figs. 1, 2), and the transition region (Fig. 2). The membrane begins at the basal body-cilium junction and runs very straight through the transition region. In longitudinal sections (Figs. 2, 3), five or six equally spaced (~245 Å space) projections (~140 Å diameter) are seen to extend from the membrane on each side of the cilium in this region. Projections on one side of the cilium are in register with corresponding projections on the opposite side (Fig. 3). Furthermore, in sections that include part of the surface of this region of membrane (Figs. 4, 5), a pattern of five or six ridges is seen. On the basis of these views, we think that the projections correspond to a set of six ridges that encircle the membrane of this part of the cilium. Throughout the main portion of the cilium, the membrane is loosely arranged around the axoneme and no surface modifications are present. Towards the end of the cilium, the membrane tapers to follow the contour of the axoneme that is gradually losing microtubules. At the extreme tip, the surface of the membrane has a specialized glycocalyx (Fig. 1). In longitudinal view, the glycocalyx appears to be composed of groups of filaments that measure up to 400 Å in length. In this view, it is similar in appearance to the ciliary crown described by Dirksen and Satir (5). Although we have examined cross sections of the ciliary tip, we have not been able to detect the ridge pattern described by others (3, 5).

Distribution of PCF

When pieces of oviduct were exposed to PCF

**Figure 1** Organization of the surface glycocalyx on the tips of oviduct cilia (arrows). Although in this view the glycocalyx resembles the ciliary crown described in mouse oviduct cilia (5), we have not seen cross sections of this region that confirm the proposed ridge-like arrangement of this surface modification. Tissue fixed in 1% glutaraldehyde and 1% tannic acid. Bar, 1,000 Å. × 35,800. Inset, × 96,900.

**Figure 2** Longitudinal section of cilia (C) showing the structure of the ciliary membrane in the transition region (T). The arrows indicate the regularly arranged surface projections found on the membrane in this region. B, basal body; mv, microvilli. Fixed in 2% glutaraldehyde. Bar, 1,000 Å. × 55,000.

**Figure 3** Longitudinal section through the base of the cilia. In this view, six regularly spaced projections can be seen extending from the membrane on each side of the cilium. Notice that the projections on one side of the cilium appear to be in register with the projections on the opposite side. Fixed in 2% glutaraldehyde plus 6% paraformaldehyde after treatment with 0.12 U/ml of neuraminidase and 0.32 mg/ml PCF. Bar, 1,000 Å. × 45,400.

**Figure 4** In this longitudinal section, the plane of section included part of the surface of the ciliary membrane. It can be seen that five to six ridges encircle the membrane and that these ridges correspond in location to the membrane projections seen in Fig. 3. Fixed with 2% glutaraldehyde plus 6% paraformaldehyde after treatment with 0.12 U/ml neuraminidase and 0.32 mg/ml PCF. Bar, 1,000 Å. × 55,700.

**Figure 5** A longitudinal section that grazed through the surface of the ciliary membrane. Here the ridges have a wavy appearance somewhat suggestive of the arrangement of the ciliary necklace found in this region (11). Fixed with 2% glutaraldehyde plus 6% paraformaldehyde after treatment with 0.12 U/ml neuraminidase and 0.32 mg/ml PCF. Bar, 1,000 Å. × 63,900.
(0.32–0.64 mg/ml), PCF was located over most of the secretory cell membrane, although the concentration of ferritin cores was variable (Fig. 7); however, the binding pattern on the ciliated cell membrane was quite distinct. On the interciliary portion of the membrane, PCF was randomly distributed with an occasional clump of ferritin on the tips of microvilli (Fig. 9); however, the density of labeling was not nearly so high as on the secretory cell membrane. PCF binding on the ciliary membrane was primarily on the tip and the transition regions of the cilium, but there was scattered, random binding to the membrane covering the main part of the cilium (Figs. 6, 8, 9).

PCF covered the tip of each cilium, usually one layer thick, in a highly reproducible fashion (Figs. 6, 8). The PCF did not bind to the outer regions of the filamentous glycocalyx but rather seemed to be enmeshed between the filaments (Fig. 8). Native ferritin (0.64 mg/ml for 10 min did not bind to the tip region (or any other part of the membrane), which rules out the possibility that PCF was passively trapped by the glycocalyx.

PCF was found to bind reproducibly to the transition region of membrane even though the concentration of bound PCF varied from cilium to cilium (Fig. 9). Figs. 10–13 are representative micrographs that show the pattern of PCF binding to this region. In longitudinal sections through the middle of the transition region, PCF was seen

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**Figure 6** Longitudinal section through cilia that had been exposed to 0.32 mg/ml of PCF for 10 min. The regular organization of PCF binding to the tip of the cilium is seen (open arrows). Furthermore, the solid arrows point to piles of PCF that characteristically bind to the main part of the ciliary membrane. Fixed with 2% glutaraldehyde. Bar, 1,000 Å. × 29,700.

**Figure 7** A view of the secretory cell surface after exposure to 0.32 mg/ml PCF for 10 min. In contrast to the ciliary membrane, this membrane is more uniformly labeled with PCF, even though there is variability in the concentration of ferritin cores. Fixed with 2% glutaraldehyde. Bar, 1,000 Å. × 34,400.

**Figure 8** A high magnification view of PCF binding to the tips of the ciliary membrane. The ordered rows of ferritin across the surface are pointed out by the open arrows. The solid arrow points to a tip cut in cross section. This shows that PCF is evenly distributed over the surface of the tip. Tissue exposed to 0.32 mg/ml PCF for 10 min and then fixed in 2% glutaraldehyde. Bar, 1,000 Å. × 63,800.

**Figure 9** Longitudinal section through the surface of a ciliated cell exposed to 0.32 mg/ml PCF for 10 min. Binding to the interciliary portions of membrane is sparse and there does not appear to be a pattern to the distribution of PCF. In contrast, PCF was regularly arranged on the portion of membrane covering the transition region of the cilia. The solid arrow points to a possible endocytic vesicle that contains ferritin. Fixed in 2% glutaraldehyde. Bar, 1,000 Å. × 39,200.

**Figure 10** A longitudinal section through the transition of the cilium. PCF is bound to membrane on both sides of the cilium. Notice that the region of PCF binding coincides with the beginning and ending of the transition region. Tissue was exposed to 0.32 mg/ml PCF for 10 min and then fixed in 2% glutaraldehyde. Bar, 1,000 Å. × 49,500.

**Figure 11** An en face view of PCF binding to the transition region of membrane. The PCF is arranged in rows across the membrane in the distal part of the transition region in this section. Tissue exposed to 0.32 mg/ml PCF for 10 min and then fixed in 2% glutaraldehyde. Bar, 1,000 Å. × 53,600.

**Figure 12** An en face view of PCF binding to the transition region of membrane. This section included the surface of the membrane covering the proximal part of the transition region, and the PCF is distributed in wavy rows. This figure and Fig. 11 together show that the arrangement of PCF binding is similar to the arrangement of surface ridges found in this segment of the ciliary membrane (Figs. 4, 5). Tissue treated the same as in Fig. 11. Bar, 1,000 Å. × 57,800.

**Figure 13** A cross section through the transition region (open arrow) that shows PCF binding to the membrane. There is a suggestion that PCF binds in juxtaposition to the attachment of the radial linkers to the membrane (small arrows). Notice that PCF is not bound to the membrane around the main part of the cilium (solid arrow). Tissue exposed to 0.32 mg/ml PCF for 10 min and then fixed with 2% glutaraldehyde. Bar, 1,000 Å. × 68,800.
bound to the membrane on both sides of the cilium (Fig. 10). Some longitudinal sections included the whole membrane of the transition region so that an en face view of PCF binding could be seen (Figs. 11, 12). In this view, PCF appeared to be scattered over the surface of the membrane; however, closer examination revealed that there was a pattern to the binding. That is, PCF seemed to be arranged in scalloped rows similar to the arrangement of the surface ridges that encircle this region. The ferritin tended to obscure the ultrastructural details of the membrane so that we could not positively determine that PCF was binding to this ridge-like surface modification. Finally, in cross sections through this region, it appears that PCF binds in relation to the "champagne-glass structures" (11) that link the doublets to the ciliary membrane (Fig. 13).

Whereas a single layer of PCF was bound to the tip and transition regions, piles of PCF were observed bound to the membrane covering the main part of the cilium (Fig. 6). There did not seem to be any pattern to the distribution of these piles of PCF; furthermore, the PCF was not associated with any surface modification of the membrane.

**Characteristics of PCF Binding**

Pieces of oviduct exposed to 0.32 mg/ml of PCF for as little as 10 s showed ferritin bound only to the tips of the cilia and to the surfaces of secretory cells. If tissue was exposed to PCF for 10 min, washed, and incubated in balanced salt solution for up to 40 min, there did not seem to be any change in the distribution of ferritin binding from that seen in tissue exposed for 10 min and fixed (Fig. 18). In contrast, in the secretory cell substantial amounts of PCF had moved off the microvilli into endocytic vesicles. Occasionally, small ferritin-containing vesicles were found in ciliated cells (Fig. 9).

Two approaches were used to look at PCF binding to nonbeating cilia. Tissue prefixed with 1% paraformaldehyde or tissue exposed to 100 μM dinitrophenol was treated with PCF (0.32 mg/ml). In both cases, PCF binding was variable. When adjacent secretory cell surfaces were coated with PCF, an indication that PCF reached the surface of the epithelium, ferritin was bound to the tips of the cilia (Fig. 17). However, rarely was ferritin seen on any other portion of the ciliary membrane or on the interciliary membrane. Dinitrophenol did not affect the distribution of PCF binding on beating cilia (see Materials and Methods).

The distribution of PCF binding varied with the concentration of PCF in the incubation medium. Binding to the tip of the cilia was observed at concentrations of 0.08 and 0.16 mg/ml. Both tip and base binding occurred at 0.16, 0.32, and 0.64 mg/ml; however, the random binding to the main portion of the ciliary membrane was most obvious at concentrations of 0.32 and 0.64 mg/ml.

**Pretreatment of Tissue**

To examine further the surface charge of ciliary membranes, we treated pieces of oviduct in several ways before exposing them to PCF. Tissue was pretreated with either trypsin, pronase, or neuraminidase. In other trials, three different molecular weights of poly-L-lysine were used to determine the effect of this cationic molecule on the binding of PCF.

Whereas trypsin (0.25%) treatment reduced the amount of binding to the membrane, pronase (1 mg/ml) completely inhibited binding. Furthermore, pronase removed the ciliary crown (Fig. 14) but did not affect the surface ridges at the base of the cilium (Fig. 15). Similarly, these proteases inhibited the binding of PCF to the secretory cell surface.

Neuraminidase at 0.12 U/ml also effectively inhibited PCF binding (Fig. 16). However, there was little inhibition when 0.06 U/ml was used. In contrast to the effects of pronase, neuraminidase did not remove the ciliary crown (Fig. 16). Like pronase, neuraminidase did not alter the surface ridges at the base of the cilia (Figs. 3-5). Neuraminidase reduced the amount of PCF binding to secretory cells.

Only poly-L-lysine with a molecular weight greater than 70,000 was effective in blocking PCF binding to the ciliary membrane. With this treatment, PCF was not observed on any portion of the ciliated cell membrane, and adjacent secretory cell membranes were also free of PCF.

**DISCUSSION**

Both colloidal iron (2, 6, 10, 17, 19, 27, 29, 30) and PCF (4, 12-14) are considered to bind specifically to sites of negative charge on surface membranes. We believe that the location of PCF on the ciliary membrane corresponds to sites of negative charge, for the following reasons: (a) PCF binding...
was localized to specific areas of the ciliary membrane that usually corresponded to regions where surface modifications were present; (b) native ferritin at concentrations of 0.64 mg/ml did not bind to the membrane; (c) the binding of PCF was inhibited by pretreatment with high molecular weight poly-L-lysine, a polycationic macromolecule that should also bind to sites of negative charge; (d) pretreatment with neuraminidase, an enzyme that removes negatively charged sialic acid residues (26), inhibited the binding of PCF.

The data suggest that these anionic sites are due to the presence of membrane glycoproteins (or mucopolysaccharides) that contain sialic acid residues because both neuraminidase and proteases removed the charge. Since neuraminidase did not remove the tip glycocalyx as did pronase, its effect probably is not due to contaminating proteases. Another possibility, that cannot entirely be ruled out, is that the charge was blocked by the binding of the enzymes to these anionic sites, thus inhibiting PCF binding (26). However, the tissue was washed before incubation in PCF, and these are conditions under which low molecular weight poly-L-lysines (<30,000) did not inhibit PCF binding. This indicates that any bound enzymes should have been removed before exposure to PCF.

As part of this study, we wanted to determine whether there were differences in the density of the negative charge at the PCF-binding sites on the ciliary membrane. To test this, we exposed oviduct epithelia to four different concentrations of PCF. Whereas little binding occurred at 0.08 mg/ml, predominantly tip binding was observed at 0.16 mg/ml. At 0.32 mg/ml, both base and tip binding occurred with occasional clumps of PCF located randomly on the shaft of the cilia. Finally, at the highest concentration, more binding occurred with occasional clumps of PCF than at the ends of the glycocalyx, the anionic sites may be either on the sides of the filamentous projections or at the surface of the membrane. This is in contrast to the rather loose arrangement of PCF binding to the membrane around the transition region of the cilia. We think that in this region the anionic sites are associated with the five or six surface ridges that encircle the membrane even though PCF did not bind to each set of ridges on any one cilium.

The number of membrane surface ridges around the transition region corresponds to the number of rows of intramembrane particles in the ciliary necklace. Furthermore, the distance between ridges (245 Å) agrees with the distance between strands (250 Å) in mammalian cilia (11). It is not unlikely that the integral proteins of the necklack contribute to the structure of the ridges. This seems to be true for mollusk ciliary membranes (9). If this is the case, then these proteins, represented by intramembrane particles in freeze-cleaved preparations, may contain the anionic sites. The random binding of PCF to the membrane of the main part of the cilium may also correspond to anionic sites associated with the scattered intramembrane particles that have been noted in this part of the membrane (11).

The distribution of negative charges on the surfaces of several types of cells (2, 13, 17, 19, 27, 29, 30) as well as on mitochondrial (14) and chromaffin granule (6) membranes has been described. Usually, the charges are distributed evenly on these membranes; however, in human fibroblasts there are discrete regions of charge separated by segments of membrane that apparently are free of any negative charge (12). In both human fibroblasts1 and baby hamster kidney (BHK) cells (13), bound PCF is cleared from the surface by a process that involves movement of ferritin into endocytic vesicles. Although in our experiment this process was observed in adjacent secretory cells, no movement of PCF on the surfaces of ciliary membranes was detected after 40 min incubation in PCF-free media. This corresponds to the fact that very little endocytosis of ferritin was detected in these cells. Therefore, the ciliary membrane appears to be relatively stable

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R. G. W. ANDERSON AND C. E. HEIN  Anionic Sites on the Oviduct Ciliary Membrane 489
due to a low rate of endocytosis-mediated membrane turnover.

It is interesting that PCF bound to ciliary membranes that had a normal beat frequency and that PCF did not inhibit beating. When beating was inhibited by treatment with either paraformaldehyde or dinitrophenol, not so many cilia bound PCF and the PCF was bound exclusively to the tip region (even after 60 min incubation in PCF). Since the secretory cells bound PCF when the ciliary beat was inhibited, it is possible that the beat had some effect on the distribution of anionic sites on the ciliary membrane.

The present study shows for the first time the existence of cell surface determinants on the oviduct ciliary membrane that are capable of interacting with the extracellular environment. Without further experimentation, it is impossible to know exactly how these determinants are important for both ciliary activity and ciliary function. In the case of negative charges on the tips of oviduct cilia, it is not unreasonable to suppose that they may be involved in the transport of the ovum. Certainly, negative charges on the surfaces of other cells (e.g., BHK cells) are involved in various aspects of cell-cell and cell-substratum interaction (13, 26). The close proximity of the ovum with its surrounding cumulus mass to the cilia during transport and the tenacity with which this complex of cells adheres to the fimbriated end of the oviduct after ovulation (1) are suggestive of some type of specific interaction. The anionic sites around the transition region of the cilium may be related to the high-affinity calcium-binding sites that have been shown to exist in this region in Paramecium cilia (8, 20).

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