Intercellular communication between epithelial and fiber cells of the eye lens

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SUMMARY

Results of electrical, dye-coupling and morphological studies have previously suggested that gap junctions mediate communication between the anterior epithelium of the lens and the underlying lens fiber cells. This connection is believed to permit ‘metabolic cooperation’ between these dissimilar cell types and may be of particular importance to the fiber cells, which are thought incapable of autonomous ionic homeostasis. We reinvestigated the nature of the connection between epithelial and fiber cells of the embryonic chicken lens using fluorescence confocal microscopy and freeze-fracture analysis. In contrast to earlier studies, our data provided no support for gap-junction-mediated transport from the lens epithelium to the fibers. Fluorescent dyes loaded biochemically into the lens epithelium were retained there for more than one hour. There was a decrease in epithelial fluorescence over this period, but this was not accompanied by an increase in fiber cell fluorescence. Diffusional modeling suggested that these data were inconsistent with the presence of extensive epithelium-fiber cell coupling, even if the observed decrease in epithelial fluorescence was attributed exclusively to the diffusion of dye into the fiber mass via gap junctions. Furthermore, the rate of loss of fluorescence from isolated epithelia was indistinguishable from that measured in whole lenses, suggesting that decreased epithelial fluorescence resulted from photobleaching and leakage of dye rather than diffusion, via gap junctions, into the fibers. Analysis of freeze-fracture replicas of plasma membranes at the epithelial-fiber cell interface failed to reveal evidence of gap-junction plaques, although evidence of endocytosis was abundant. These studies were done under conditions where the location of the fracture plane was unambiguous and where gap junctions could be observed in the lateral membranes of neighboring epithelial and fiber cells. Paradoxically, tracer molecules injected into the fiber mass were able to pass into the epithelium via a pathway that was not blocked by incubation at 4°C or by treatment with octanol and which excluded large (~10 kDa) molecular mass tracers. Together with previous measurements of electrical coupling between fiber cells and epithelial cells, these data indicate the presence of a low-resistance pathway connecting these cell types that is not mediated by classical gap junctions.

Key words: lens, confocal microscopy, gap junction, intercellular communication, freeze-fracture

INTRODUCTION

The lens of the eye is composed of elongated fiber cells bounded at their anterior ends by an epithelial monolayer (Fig. 1). In young embryos, the lens consists of primary fiber cells, each extending from the apical surface of the epithelium to the posterior lens capsule (Fig. 1A). Secondary fiber cells are produced subsequently by the differentiation of epithelial cells near the lens equator. Newly formed fiber cells are laid down over the pre-existing fibers. In older lenses, therefore, only the outermost fibers make direct contact with the epithelium (Fig. 1B).

It is widely accepted that the epithelium plays an important role in regulating the passage of ions and metabolites between the aqueous humor of the eye and the cytoplasm of the lens fiber cells (Kinsey and Reddy, 1965). Goodenough et al. (1980) proposed that ions and metabolites gain access to the fiber cell cytoplasm via gap junctions connecting the closely apposed apical membranes of the epithelial and fiber cells. This ‘metabolic cooperation’ could provide essential support for the terminally differentiated fiber cells, many of which lack cellular organelles (Goodenough et al., 1980).

There is strong evidence that lens fiber cells are linked to each other by an extensive network of low resistance junctions (for a recent review of cell communication in the lens, see Mathias and Rae, 1989) that are functionally analogous to gap junctions characterized in other tissues. Fiber-fiber junctions have been studied using electrical (Duncan, 1969; Eisenberg and Rae, 1976), morphological (Kuszak et al., 1978, 1980; Lo and Harding, 1986), dye tracer (Rae, 1974; Miller and Goodenough, 1986) and reconstitution techniques (Peracchia et al., 1985). Likewise, lens epithelial cells appear to be coupled to
their neighbors by gap junctions (Miller and Goodenough, 1986; Duncan et al., 1988; Jacob, 1988; Stewart et al., 1988; Cooper et al., 1989). A patent connection between the epithelium and the underlying fiber cells has been proposed on the basis of electrical coupling (Rae and Kuszak, 1983), dye transfer (Miller and Goodenough, 1986) and freeze-fracture experiments (Goodenough et al., 1980; Miller and Goodenough, 1986). Consequently, the hypothesis that normal fiber cell functions are maintained via a direct connection to the epithelium is widely accepted and has prompted numerous studies of the role of epithelial cell dysfunction in cataract formation.

There are, however, observations that cast doubt on the direct coupling, metabolic or otherwise, of epithelial to fiber cells in the lens. For example, it is difficult to envisage how gap junctions are formed and maintained between fibers and epithelial cells, where cell contacts are continuously broken by the interposition of newly differentiated fiber cells. In contrast to the studies reviewed above, a recent freeze-fracture EM study of the apical membranes of cells at the epithelium-fiber interface (EFI) of adult chicken lenses revealed a striking paucity of intra-membrane particle assemblies (gap-junctional plaques) that might mediate communication between epithelial and fiber cells (Brown et al., 1990).

To investigate further the nature of the connection between epithelial and fiber cells, we performed electron microscopic freeze-fracture analysis of the EFI and laser scanning confocal microscopy of living, dye-loaded lenses. These studies revealed no appreciable transfer of small molecules from epithelial to fiber cells. Surprisingly, low-molecular-mass tracers did pass in the opposite direction, from dye-injected fibers to overlying epithelial cells. This transfer occurred between cells lacking any detectable gap-junction assemblies.

**MATERIALS AND METHODS**

**Solutions and reagents**

Measurements were made at room temperature on lenses maintained in artificial aqueous humor (AAH; for composition see Bassnett et al., 1990). The dyes used in this study were carboxyfluorescein, carboxyfluorescein-diacetate, carboxy-seminaphthorhodafluor-1-acetoxyethyl-diacetate (SNARF-1-AM), 2,7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) and dextran-tetramethylrhodamine (dextran-rhodamine; anionic, Mr 10,000). Dyes were obtained from Molecular Probes (Eugene, OR) and, with the exception of dextran-rhodamine (which was stored as an aqueous stock), were stored at −20°C as stock solutions in dimethylsulfoxide. Unless indicated, other reagents were purchased from Sigma Chemical Co. (St Louis, MO).

**Animals**

Fertilized chicken eggs were obtained from Truslow Farms (Chester-town, MD) and incubated at 38°C. Lenses were removed from embryos at day 6 or day 15 of development (E6 or E15) by making an incision in the posterior globe and removing the vitreous body and attached lens with fine forceps. In some cases, the lens epithelium was separated from the fiber mass and viewed en face by pinning it to the surface of a Petri dish as described by Bassnett (1990).

**Dye loading**

Esterified dye derivatives were introduced into the lens epithelium by incubating the lens, with its attached vitreous body, for 15 minutes in AAH containing 10 μM dye. Lenses were then washed in AAH and viewed. To follow the transfer of dye from the epithelium to the fiber cells over time, lenses were incubated in 30 μM carboxyfluorescein diacetate for 3 minutes, washed in AAH and examined on the confocal microscope. In these studies the vitreous body, which might have otherwise constituted a reservoir of esterified dye, was carefully removed before the lens was viewed. Lenses were examined in a chamber constructed from a 35 mm plastic Petri dish with a viewing window cut in its base. A coverslip was glued across the window using silicone adhesive. The first images of the lens were recorded 5 minutes after beginning the dye loading. To confirm that the photodetection system gave a linear response across the measured range and to estimate intracellular dye concentration, the confocal viewing system was calibrated using solutions of carboxyfluorescein. The carboxyfluorescein was first dissolved in dimethylsulfoxide at 1 mM, then diluted into buffer containing 120 mM KCl, 20 mM NaCl and 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (pH 7.0). Calibration measurements were made at room temperature on dye samples held in Cunningham chambers (Bassnett et al., 1990) using a ×20 objective. The laser intensity and the gain and black level settings on the photomultiplier tubes were the same as those used for measurements on the lens.
Dye injection

Lenses were injected iontophoretically with solutions containing 0.4 mM dextran-rhodamine, 0.1 mM carboxyfluorescein, or both. The injection pipettes were pulled from 1.2 mm o.d. filamented borosilicate glass (WPI Inc., Sarasota, FL) and, if filled with 3 M KCl, had a resistance of ~10 MΩ. The tip and shank of the pipette were filled with the dye solution. The remainder of the pipette barrel and the body of the pipette holder were filled with 3 M KCl. Pipettes were inserted into the back of the lens through the posterior capsule. To stabilize lenses during impalement and injection they were first embedded in a 3% gel of low melting point agarose (LMP-agarose; BRL, Life Technologies Inc., Gaithersburg, MD) dissolved in AAH. The gelling temperature of LMP-agarose is ~30°C, enabling the lens and attached vitreous body to be suspended in a drop of molten agarose without thermal damage. The lens was moved into the desired orientation and the agarose allowed to gel at room temperature before being overlaid with AAH. The preparation was then moved to the stage of the microscope where a Ag/AgCl ground return was placed in the viewing dish. The injection pipette was connected to a model 707 microprobe system (WPI, Inc.) and iontophoretic injection parameters controlled from a function generator (Wavetek, San Diego, CA). Dye was injected by passing 20 nA hyperpolarizing current pulses at 1 Hz for 15 minutes.

Confocal microscopy

Lenses were viewed on an inverted microscope (Axiovert-35, Zeiss) equipped with a laser scanning confocal imaging system (MRC-500, Bio-Rad). For most experiments, a fluorescein filter set, consisting of a 488±10 nm excitation filter, a 510 nm long pass dichroic reflector and a 515 nm long pass emission filter, was used. For the dye injection experiments, it was sometimes necessary to differentiate between carboxyfluorescein and dextran-rhodamine fluorescence. In this case, sequential images were obtained using the fluorescein filter set and a rhodamine filter set (514±10 nm excitation filter, 540 nm long pass dichroic reflector and 550 nm long pass emission filter). The two images were false color-coded and merged into a final image using software supplied with the MRC-500. The microscope was also fitted with a transmitted light detector enabling a non-confocal, phase-contrast image to be collected in register with the confocal fluorescence image. For most applications, a plan-NeoFluar ×20 objective (NA 0.5; Zeiss) was used but, for detailed examination of the fluorescence distribution within individual epithelial cells, a ×63 plan-Apochromat oil-immersion objective was used (NA 1.4; Zeiss). Sample fluorescence and transmitted light were detected by means of photomultiplier tube detectors. For quantitative fluorescence measurements, gain and black level settings were set manually. In the MRC-500 system, a variable aperture controls the thickness of the optical sections. Measurements were always made with this aperture set at the minimum diameter. Final images were produced by averaging 10-20 frames.

Image analysis

To quantify the diffusion of carboxyfluorescein from the epithelium to the fibers, pixel intensity profiles were obtained along a line that was nine pixels wide and oriented perpendicular to the surface of the lens epithelium. This technique allowed a degree of signal averaging without seriously degrading the spatial resolution. By using the same coordinates at successive time points, the change in lens cell fluorescence could be measured with time.

Freeze-fracture

A detailed description of the freeze-fracture procedure has been published previously (Brown et al., 1990). Briefly, lenses were fixed in a solution of 2.5% glutaraldehyde in 0.07 M sodium cacodylate buffer (pH 7.2) and subsequently cryo-protected with 25% glycerol in cacodylate buffer. Lenses were snap-frozen in super-cooled liquid nitrogen. Fracture and replication were performed at ~170°C in a Balzers 400T freeze-etch unit. Replicas were retrieved onto Formvar-coated grids and examined on a JEOL JEM 1200EX transmission electron microscope operated at 80 kV.

At the anterior surface of the lens, the apical membranes of the epithelial cells abut those of the elongating fiber cells. The close apposition of the apical membranes of these two cell types constitutes a unique interface in the lens that has been referred to as the epithelial/fiber interface (EFI; Brown et al., 1990). To expose the maximum area ofEFI by freeze-fracture, the lens was fractured at an acute angle to its anterior surface. With this orientation the fracture plane was likely to pass along the EFI rather than perpendicular to it. Even with this fracture orientation, however, some portions of the lateral membranes of epithelial and fiber cells were likely to be present in the replica in addition to the EFI. The approach used to clearly discriminate the fractured apical membranes of the EFI from basal epithelial membranes and lateral membranes of fiber and epithelial cells is given below.

At low magnification the edge of the lens was identified and the epithelial layer located. The epithelial layer was recognizable by the presence of nuclei and other cytoplasmic organelles and the convoluted appearance of the apico-lateral membranes. One side of the epithelium was bounded by frozen buffer, characterized by the absence of any organized structures and the fine granular appearance of the replica. On the other side of the epithelium, the apical tips of the fiber cells were recognized by the paucity of organelles in comparison to the cytoplasm of the epithelial cells. At lower magnification, the cross-fractured tips of the fibers were seen to be contiguous with fiber cells of the superficial cortex that were readily identified by their columnar organization and hexagonal cross-sections. The EFI was located between fractured epithelial and fiber cells. The apical membranes of the epithelial and elongating fiber cells at the EFI were identifiable by their surface topology and matched closely those described earlier using SEM (Kuszak and Rae, 1982; Rae and Kuszak, 1983; Willekens and Vrensen, 1985). To ensure that the fracture plane passed from within the fiber cells, up through the epithelium and into the buffer rather than jumping back and forth between the various cell layers, we generated stereo pairs of the replica at each interface between the different layers. These three-dimensional views demonstrated that the fracture plane moved in a step-wise manner through the tissue, thus simplifying the identification of the fractured surfaces. It is conceivable that a fracture passing between the basal membrane of the epithelial cells and the anterior lens capsule might resemble the EFI. However, in the replicas used here, the EFI was always bounded on one side by epithelial cells and on the other by fibers. Only for fractured apical membranes (i.e. authentic EFI) would these conditions be met. In summary, freeze-fracture replicas of the anterior lens contain sufficient morphological cues to allow the unambiguous identification of the EFI.

RESULTS

Dye loading

When whole E6 lenses were incubated for 15 minutes in AAH containing 10 μM carboxyfluorescein diacetate, fluorescence was largely restricted to a narrow band at the anterior surface of the tissue, corresponding to the lens epithelium (Fig. 2). With this dye, fluorescence results from the activity of intracellular esterases. These enzymes cleave the cell-permeant carboxyfluorescein-diacetate to generate relatively impermeant carboxyfluorescein. At E6, the extracellular space of the lens is readily accessible to small molecules (unpublished observation). Therefore, the restriction of intracellular fluorescence to the epithelium probably reflected the distribution of esterase
Fig. 2. Merged transmitted light (blue) and confocal fluorescence (red) images of an E6 lens following a 15 minute incubation in carboxyfluorescein diacetate. Note that the fluorescent dye is restricted to the lens epithelium. Lens epithelium (LE), lens fibers (LF), ciliary epithelium (CE), vitreous humor (V). Bar, 300 μm.

Fig. 3. Montage of confocal images of an E6 lens obtained at 5 minute intervals following a 3 minute incubation in 30 μM carboxyfluorescein diacetate. (A) Shows the position of the line segment used for generating the pixel intensity profile shown in Fig. 4.
activity rather than lack of access of dye to the inner cells. In the thickened annular pad near the lens equator, fluorescence was less intense and the underlying fiber cells were also slightly fluorescent.

Carboxyfluorescein is a relatively small molecule ($M_r$ 376), well below the size exclusion limit for gap junction systems (Simpson et al., 1977). The finding that, after a period of 15-20 minutes, dye was restricted to the anterior epithelium suggested that the epithelium and underlying fiber cells were not well-coupled. To determine whether other dyes behaved in a similar fashion, lenses were loaded with the membrane-permeant esters of two other small fluorescent molecules, BCECF ($M_r$ 520) and SNARF-1 ($M_r$ 453). As with carboxyfluorescein, confocal microscopy revealed that these dyes were also restricted to the anterior lens epithelium (data not shown).

To ensure that the localization of fluorescence in the central epithelium did not result from damage to the epithelium-fiber interface during dissection, lenses were loaded with dye while still in the eye. In these instances, eyes were removed from the embryos and a small incision made in the posterior globes. Whole eyes were then incubated in carboxyfluorescein-diacetate. Following a 15 minute loading period, lenses were removed and examined as quickly as possible (<2 minutes). The distribution of fluorescence within the central epithelium of lenses prepared in this fashion was qualitatively similar to that observed in vitro.

The restricted distribution of fluorescent dyes might have resulted from binding to cellular components or sequestration...
in compartments within the epithelial cells. To investigate this possibility, the confocal microscope was used to visualize, en face, carboxyfluorescein-loaded epithelial cells in place on the lens. By employing a ×63 objective it was possible to verify that the dye was evenly distributed throughout the cytoplasm, including the nucleus, and was not contained within discrete subcellular compartments (data not shown).

It was possible that incubation in the cell-permeant dye caused gap junction closure, perhaps due to the byproducts of the de-esterification reaction. To test this we loaded a mechanically isolated epithelium with Snarf-1-AM and subsequently injected carboxyfluorescein into a cell in the epithelium. The carboxyfluorescein fluorescence rapidly spread to neighboring cells, indicating that the dye loading process had not caused epithelial cells to uncouple from each other (data not shown).

An estimate of the degree to which epithelium and fiber cells were dye-coupled could be obtained by monitoring the loss of fluorescence from the epithelial cells over time and the concomitant appearance of fluorescence in the nearby fiber cells. Fig. 3 shows a sequence of confocal images taken at 5 minute intervals, demonstrating the change in fluorescence distribution with time. The initial intracellular dye concentration in the epithelium was obtained by reference to calibration curves generated in Cunningham chambers. With the loading protocol used, the initial intracellular dye concentration was 20–40 μM. The most striking feature of the montage shown in Fig. 3 is that, although there was a gradual loss of fluorescence, the specific labelling of the epithelium was still evident an hour after the original loading period. Pixel intensity profiles were obtained along the line segment indicated in Fig. 3 for sequential images. The change in pixel intensity in the epithelial and fiber cells over time is shown in Fig. 4.

To help interpret these data, we simulated the temporal and spatial dependence of the fluorescence signals using a diffusional model (Fig. 5). The diffusion of fluorescent dye molecules from the epithelium was modeled using Einstein’s model (Fig. 5). The diffusion of fluorescent molecules into the model space. In each case dye was confined initially to the outer 10 μm of tissue (corresponding to the epithelial layer) and subsequently permitted to diffuse into the fiber cells. In the simplest models (Fig. 5A-C), no barrier to diffusion was interposed between the epithelial and fiber cells. In such models, the step function fluorescence profile measured in the first 5 minutes spread into a Gaussian that increased in width and decreased in amplitude over time at a rate that depended on the diffusion coefficient assigned to the dye in the lens cell cytoplasm.

Although the decrease in the amplitude of the fluorescence in the epithelium could be simulated using simple diffusional models (see Fig. 5C), there were two major discrepancies. First, the calculated dye diffusion coefficient necessary to simulate the measured kinetics (Fig. 4) was unreasonably small (1×10⁻¹³ m² s⁻¹). A diffusion coefficient of the order of 1×10⁻¹¹ m²s⁻¹ was expected, based on the diffusion of comparable compounds. For example, a value of 2.6×10⁻¹¹ m²s⁻¹ was calculated for the diffusion coefficient of lactic acid in lens fiber cell cytoplasm (Bassnett et al., 1987). Second, simple diffusional models predicted the presence of a relatively high concentration of dye molecules in fiber cells immediately adjacent to the epithelium with no discontinuity between the two layers. However, the experimental observations indicated a near-constant concentration of dye molecules along the length of the

\[
\langle x^2 \rangle = \frac{2k_B T}{f} t,
\]

where \( f = 6 \pi \eta a \), and \( a \) is the radius of the spherical diffusing particle and \( \eta \) is the viscosity of the solvent. Boltzmann’s constant is \( k_B \), the temperature is \( T \) and the time is \( t \). The time dependence of a concentration gradient then follows Fick’s first law. In the models we used one-dimensional diffusion. Since the confocal microscope probed a small number of cells surrounded by a large number of similar cells, the diffusion of the fluorescent molecules out of the one-dimensional model into the other dimensions was balanced by the diffusion of fluorescent molecules into the model space. In each case dye was confined initially to the outer 10 μm of tissue (corresponding to the epithelial layer) and subsequently permitted to diffuse into the fiber cells. In the simplest models (Fig. 5A-C), no barrier to diffusion was interposed between the epithelial and fiber cells. In such models, the step function fluorescence profile measured in the first 5 minutes spread into a Gaussian that increased in width and decreased in amplitude over time at a rate that depended on the diffusion coefficient assigned to the dye in the lens cell cytoplasm.

Although the decrease in the amplitude of the fluorescence...
fiber cells. There was also a significant decrease in fluorescence between the epithelium and the fiber cells.

To account for these features, the diffusional model was modified by interposing a partial barrier between the epithelial and fiber cells (Fig. 5D-L). In reality, this might be the juxtaposed apical membranes of the two cell types. Passage of dye molecules across this barrier was assumed to occur by passive diffusion through gap-junction channels that occupied some fractional area of the apical cell membrane. In Fig. 5, panels D-L show a matrix of diffusion profile simulations where both the diffusion coefficient and the fractional area of apical membrane present in the form of gap junction channels have been varied. If the diffusion coefficient of carboxyfluorescein was close to that of lactate in this tissue (~$10^{-11}$ m$^2$ s$^{-1}$; Bassnett et al., 1987), then a value of 0.1% (Fig. 5L) for the fractional area most closely simulated the experimental data.

If the fractional area was allowed to increase above 0.1%, the dye diffusion coefficients in the two cell types necessarily differed. At a value of 10%, for example, the calculated diffusion coefficients had to differ by two orders of magnitude to simulate the experimental data (simulations not shown). Such a difference did not seem reasonable.

We also considered more exotic models, such as the bulk flow of material from the fibers to the epithelium. The near-uniform distribution of fluorescence along the length of the fiber cells argued against such models.

The models described above assumed that the decrease in epithelial fluorescence was entirely due to passive diffusion of dye into the fiber cells ($k_{2}$ in Fig. 6). As shown in Fig. 6,
however, there are several other pathways that might account for this decrease. For example, dye might diffuse into neighboring epithelial cells through gap junctions in the lateral membranes ($k_{11}$). As the central epithelium was uniformly labelled (Fig. 2), lateral diffusion would not result in net loss of dye molecules from a given cell (although a very slow loss of dye into the fiber cells through the bow region would be expected). Loss of epithelial fluorescence due to the combined effect of photobleaching of the dye and leakage of dye into the bath ($k_B + k_L$) probably constituted a more significant pathway, particularly for cells in the central epithelium. This component was estimated empirically by monitoring the loss of fluorescence from epithelia that had been detached from the fiber mass. Sheets of mechanically isolated lens epithelial cells remain viable and have been utilized previously for physiological studies (Bassnett, 1990). In this culture system, the close apposition of the apical membranes of the epithelial cells to the surface of the Petri dish mimicked the situation in the lens, where the cleft between the epithelium and the fibers is narrow. A series of images was obtained under viewing conditions identical to those used for whole lens measurements. Fig. 7 shows fluorescence measurements obtained from the central regions of four isolated epithelia and the integrated fluorescence intensity from the central epithelium replotted from

![Fig. 9. High-magnification freeze-fracture electron micrograph revealing E-face (A) and P-face (B) exposures of membranes at the EFI of an E15 lens and representative gap junctions conjoining the lateral membranes of lens epithelial cells (C) and fiber cells (D). At the EFI, both large (filled arrows) and small (open arrows) vesicles are evident. Note the tight packing of the intramembrane particles of lens epithelial gap junctions (C) with the loose and random particle packing in the fiber cell gap junctions (D). Bars, 200 nm.](image-url)
The curves may be fitted by an exponential increase, followed by an exponential decay, corresponding to the activation of the dye by intracellular esterases and subsequent loss via one or more of the pathways outlined in Fig. 6. A least-squares routine was used to fit the following equation to the data:

$$I(t) = A \left[ \exp(-\tau_d t) - \exp(-\tau_r t) \right],$$

where the free parameters were: $A$, the relative amplitude of the fluorescence, $\tau_d$ the decay time of the fluorescence signal, and $\tau_r$ the rise time of the fluorescence signal. The decay times for both signals were very similar. For the isolated epithelium $\tau_d = 0.00059 \text{ s}^{-1}$ and for the attached epithelium $\tau_d = 0.00060 \text{ s}^{-1}$. By comparing scanned and unscanned regions of the isolated epithelia at the end of the experiment, it was estimated that approximately 30% of the decrease in fluorescence was caused by photobleaching of the dye. The remainder of the fluorescence loss was presumably due to diffusion into the bath ($k_L$).

**Freeze-fracture**

Freeze-fracture analysis was undertaken to determine the distribution and abundance of gap junctions in the apical membranes of epithelial and fiber cells at the EFI. Lenses from both E6 and E15 embryos were examined. Fig. 8 shows a low-
replicas of more than 20,000 included for comparison. Despite examining membrane the presence of gap junctions in this study (the data for the adjacent to the EFI.

epithelial (Fig. 9C) and fiber cells (Fig. 9D) immediately junctions were often observed in the lateral membranes of blies or gap-junction plaques of any size. In contrast, gap at E6 and E15, there was no indication of loose particle assem-

ification images of the E and P faces of a freeze-fracture replica of an E6 lens. By careful orientation of the tissue in the fracture device, it was often possible to obtain replicas of the apical membranes of several contiguous cells. Higher-magnification images of the E and P faces of a freeze-fracture replica are shown in Fig. 9A and B, respectively. Although large numbers of individual intra-membrane particles were evident at E6 and E15, there was no indication of loose particle assem-

blies or gap-junction plaques of any size. In contrast, gap junctions were often observed in the lateral membranes of epithelial (Fig. 9C) and fiber cells (Fig. 9D) immediately adjacent to the EFI.

Table 1 indicates the area of apical membrane examined for the presence of gap junctions in this study (the data for the adult chicken lenses are from Brown et al. (1990) and are included for comparison). Despite examining membrane replicas of more than 20,000 µm² of apical membrane, only two small gap junctions were observed in these preparations (Table 1). In E15 lenses it was possible to obtain replicas of membrane faces from the EFI in the annular pad region in addition to the central epithelium. No gap junctions were observed at the EFI in either the central or annular pad regions of the E15 lenses. At either age, the predominant feature of the membrane replicas was the presence of numerous vesicular events (Fig. 9A and Table 2). As noted previously (Brown et al., 1990), these fell into two size classes, large vesicles with diameters of approximately 130 nm and smaller vesicles with diameters of about 45 nm. Table 2 shows the size distribution of endocytotic profiles at the EFI in E6, E15 and adult lenses (the latter data are taken from Brown et al., 1990). Endocytotic profiles from the EFI were consistent in both size and abundance in lenses from embryos and adults.

**Dye injection**

Previous workers demonstrated dye communication between fiber cells and epithelial cells by iontophoretic injection of dye into the fiber cells (Miller and Goodenough, 1986). As these data appeared incompatible with the results presented above, we studied the diffusion of dye following microinjection into lens fiber cells.

Co-injection of dextran-rhodamine and carboxyfluorescein revealed different patterns of dye distribution. Both dyes spread rapidly anteriorly and posteriorly in the fiber cells from the injection point. However, the distribution of dextran-rhodamine was sharply defined, outlining one or a few fiber cells, while carboxyfluorescein spread laterally in a diffuse column many cells wide. The different distribution of the dyes was probably related to their molecular mass. Dextran-rhodamine ($M_r \sim 10,000$) was presumably unable to pass through gap junctions linking fiber cells whereas carboxyfluorescein ($M_r 376$) was free to diffuse from fiber to fiber.

Fig. 10 shows the behavior of the two dyes at the EFI in the central region of the lens. Dextran-rhodamine remained in the injected fiber(s) while carboxyfluorescein diffused into the overlying epithelial cells. Occasionally, it appeared as if a small amount of rhodamine dextran was able to pass into the epithelium. However, this observation was the result of bleed-

through from the fluorescein channel of the microscope into the rhodamine channel. When rhodamine dextran was injected alone into the fibers, diffusion of the dye into the epithilium was not observed (data not shown).

We confirmed the observations made on the confocal micro-

scope by two other visualization techniques. First, we repeated the studies of Miller and Goodenough (1986), by injecting Lucifer Yellow into the fibers, then fixing and sectioning the lens. Our results confirmed their earlier observations showing that dye spread from the fiber cells into the epithelial cells (data not shown). Second, we injected carboxyfluorescein into the fiber cells, stripped away the epithelium and viewed the epithelial sheet en face. A small patch of fluorescent cells was always observed in the epithelium (Fig. 11). To investigate whether fiber-epithelial coupling was developmentally regulated, we injected lenses ranging from E6-E15. In lenses from older embryos, dye injected into the central region was never detected in the epithelium. However, histological examination revealed that the dye diffused widely from the point of injection, dissipating as it passed through the layers of secondary fiber cells. By inserting the pipette into outer fiber cells that were in direct contact with the epithelium, it was possible to observe transfer of dye to the epithelium in all cases. Thus, there was a patent connection between fibers and epithelial cells in lenses from embryos at least as old as E15 (data not shown).

Additional experiments were performed to define the nature of the connection between the fiber cells and the epithelium. In each case, dye transfer was assayed by injecting carboxyfluorescein into the fiber mass, removing the epithelium and examining it en face. In four experiments, dye transfer was not blocked by a 15 minute pre-treatment of the whole lens with 1 mM octanol, a procedure known to close epithelial gap junctions in frog and human lenses (Duncan et al., 1988; Stewart et al., 1988). In each case, dye diffused readily to adjacent fiber cells, indicating that fiber-fiber junctions were also insensitive to this treatment.

It was conceivable that dye was transferred between fibers and epithelia via an extracellular pathway. This would require exocytosis of dye from the apical tips of the fiber cells into the sub-epithelial space and subsequent endocytotic uptake by the

### Table 1. Analysis of gap junctions on elongating fiber cell apical membranes

<table>
<thead>
<tr>
<th></th>
<th>E6 lens</th>
<th>E15 lens</th>
<th>*Adult lens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area of fiber cell membrane examined (µm²)</td>
<td>7,404</td>
<td>2,760</td>
<td>12,650</td>
</tr>
<tr>
<td>Number of gap junctions observed</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Average size of gap junctions (µm²)</td>
<td>0.03</td>
<td>–</td>
<td>0.25</td>
</tr>
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</table>

*Data from Brown et al. (1990).

### Table 2. Analysis of vesicle profiles on elongating fiber cell apical membranes

<table>
<thead>
<tr>
<th></th>
<th>E6 lens</th>
<th>E15 lens</th>
<th>*Adult lens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large vesicles</td>
<td>3,977</td>
<td>1,533</td>
<td>9,366</td>
</tr>
<tr>
<td>Small vesicles</td>
<td>5,624</td>
<td>2,088</td>
<td>7,111</td>
</tr>
<tr>
<td>Large vesicle diameter (nm)</td>
<td>123±26.7</td>
<td>126±18.2</td>
<td>125±20.7</td>
</tr>
<tr>
<td>Small vesicle diameter (nm)</td>
<td>44±8.1</td>
<td>42±4.0</td>
<td>45±4.9</td>
</tr>
<tr>
<td>Large vesicles/µm² membrane</td>
<td>0.54</td>
<td>0.56</td>
<td>0.74</td>
</tr>
<tr>
<td>Small vesicles/µm² membrane</td>
<td>0.76</td>
<td>0.76</td>
<td>0.56</td>
</tr>
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</table>

*Data from Brown et al. (1990).
apical membranes of the epithelial cells. Freeze-fracture replicas provided evidence for such activity in this region (Fig. 9A). To determine whether epithelial cells accumulated dye by endocytosis, whole lenses were incubated in 0.1 mM CF for 15 minutes. Previous confocal microscopy studies revealed that extracellular dye was able to permeate the subepithelial space in these lenses (unpublished observation). There was no indication of any accumulation of fluorescence in epithelium isolated following this procedure. Endocytosis is an active metabolic process that is inhibited at low temperature. Injection of dye and subsequent dissection and viewing of the epithelium were, therefore, performed at 4°C to determine whether dye transfer was inhibited at low temperature. In three experiments, there was no qualitative difference between the transfer of dye at room temperature or 4°C. These experiments indicated that dye transfer was not achieved by a coupling of exocytosis and endocytosis or other energy-dependent pathways.

**DISCUSSION**

**Dye loading**

The major finding of the dye-loading experiments was that we were unable to detect a patent intercellular pathway from lens epithelial cells to the underlying fiber cells. Dye loaded 'biochemically' into the lens epithelium was retained there for more than 1 hour. We have also observed this phenomenon in rat lenses (unpublished observation) and Wolosin et al. (1988) have noted a similar distribution in dye-loaded amphibian lenses. Miller and Goodenough (1986) demonstrated that dye injected into epithelial cells was free to diffuse within the epithelium but was not detectable in the fiber cells. They suggested that dye diffusing into the fiber cells was likely to be diluted to below the limit of detection. This is a difficult problem in the lens, where the two cellular compartments (epithelium and fiber cell mass) are of such different volumes. In the present study, rather than attempting to quantify the appearance of dye in the fibers, loss of dye from the epithelium was monitored. Modeling studies suggested that the prolonged retention of dye in the epithelium was incompatible with the presence of abundant epithelial-fiber junctions. Microscopic examination revealed no evidence of compartmentalization of the dye that might have restricted its diffusion. It is unlikely that the dye was bound significantly to submicroscopic components within the cells, as iontophotically injected carboxyfluorescein diffused readily through the cytoplasm of the fiber cells.

One possible objection to the use of esterified dyes for 'biochemically' loading the lens epithelium is that some byproduct, capable of blocking junctional communication, could be produced by intracellular hydrolysis of the ester linkage required to generate the trapped fluorescent probe. De-esterification of one mole of carboxyfluorescein-diacetate generates two moles of acetic acid, a normal metabolite. The final intracellular dye concentration was 20-40 μM, leading to the release of sufficient acetic acid to increase the cytoplasmic concentration by 40-80 μM. If intracellular pH was significantly lowered by the production of acetic acid, it is conceivable that communication through pH-sensitive gap junctions could be affected. Bassnett (1990) calculated the buffering capacity of chicken embryo lens epithelial cells maintained in AAH to be 74.5 mM. Given this level of buffering, even in the absence of other intracellular pH regulatory mechanisms, the additional acid load imposed by de-esterification of the dye would have a negligible effect on epithelial pH. In addition, carboxyfluorescein loaded using the de-esterification procedure has been used successfully to study cell-cell communication in other tissues using the FRAP (Fluorescence Recovery After Photo-bleaching) technique (Wade et al., 1986; Anders, 1988).

To determine directly whether intracellular de-esterification of dyes leads to the closure of gap junctions we injected carboxyfluorescein into epithelial cells that had previously been loaded with carboxy-Snarf-1. The fact that the injected dye was able to diffuse into neighboring epithelial cells suggests that, in the epithelium at least, the de-esterification process does not lead to uncoupling of the cells. We conclude that dye loaded biochemically into the epithelium would be free to diffuse into the fibers if the two cell types were connected by communicating junctions.

**Freeze-fracture**

The dye-loading experiments provided evidence that epithelial and fiber cells were, at best, weakly coupled. In light of this, the absence of junction plaques in the freeze-fracture replicas was not surprising. The present freeze-fracture observations were consistent with those reported previously in the adult chicken lens (Brown et al., 1990) where there was less than one gap-junction plaque present in every 100 cell apices examined. In earlier studies, Goodenough et al. (1980) and Miller and Goodenough (1986) reported epithelial-fiber cell junctions in the adult and embryonic chicken lens, respectively, although they did not comment on the relative abundance of the junctions. The reason for the discrepancy between these and the present data is not clear. In this study, we were able to detect gap junctions in the lateral membranes of epithelial and fiber cells but, when it could be verified that the fracture plane passed through the membranes at the EFI, gap junctions were absent.

**Dye injection**

Miller and Goodenough (1986) demonstrated that dye, iontophotically injected into the fiber cells, passed readily into the epithelium. Treatment with CO₂ to lower pH, did not block dye transfer. Rae and Kuszaak (1983) passed current into fiber cells and detected corresponding voltage transients in the overlying epithelium. By changing the relative positions of the stimulating and recording electrodes they were able to show that current passed directly from the fibers into the epithelium and not via a more circuitous path through the lens equator. In the present study, we confirmed the presence of a diffusional pathway from the fibers to the epithelial cells. This pathway was insensitive to treatment with octanol and low temperature, and discriminated between large and small fluorescent markers (it should be noted that octanol also failed to uncouple fiber cells, which are believed to be linked by gap junctions). With the exception of the insensitivity to octanol, these are precisely the characteristics expected of gap junction-mediated communication and appear, at first sight, to be incompatible with the dye-loading and morphological data presented above. There are several possible explanations for the different results obtained using the two approaches. Previous studies have
generally demonstrated flow of current or dye from fiber cells to epithelial cells, not in the reverse direction. It is conceivable, therefore, that the junctions at the EFI are strongly rectifying, permitting communication in one direction only. Another possibility is that there is a continuous movement of fluid from the fibers to the epithelium, effectively preventing diffusible molecules from passing from the epithelial cells to the fiber cells. It is also possible that current flowing during iontophoresis induces the formation of a latent cell-cell communication. Finally, the dye-injection protocol may be a more sensitive measure of cell-cell coupling than the dye-loading method. Under these circumstances, it is possible that a small amount of junction-mediated diffusion occurs and that this was only detected by the dye-injection approach. However, this does not explain the absence of junctional structures in the freeze-fracture replicas.

If the transfer of dye from fibers to epithelial cells is not mediated by typical gap junctions, but by another pathway, this may have implications for other biological systems. For example, it is known that lens epithelial cells and other eucaryotic cells may be electrically coupled but not dye coupled (Duncan et al., 1988). A new type of cell-cell communication pathway, with a selectivity different from typical gap junctions, might account for these observations.

Physiological consequences

It has long been accepted that lens fiber cells are nourished and their ionic composition maintained by diffusion of ions and metabolites from the overlying epithelium (Goodenough et al., 1980). Using microelectrode techniques, Duncan (1969) demonstrated that intra-lenticular electrical resistance is negligible compared to that of the superficial membranes of the lens. Thus, in many respects, the bulk of the lens constitutes a syncytium, where gaining access to the fiber cell cytoplasm is likely to be a rate-limiting step in ion transport and the delivery of nutrients to the inner cells. The present data suggest that the diffusion of low molecular mass markers from the epithelium to the fibers must be extremely slow, if it occurs at all. It seems unlikely, therefore, that this pathway constitutes the major route by which metabolites enter the rapidly growing lens fibers. Our recent studies showed that superficial lens fibers contain many of the organelles necessary for normal cellular function (Bassnett, 1992; Bassnett and Beebe, 1992). Dye-permeation studies have shown that extracellular tracers have ready access to the inner fiber cell membranes (Bassnett and Beebe, unpublished observation). Endocytosis is a prominent feature of freeze-fracture replicas of lens fiber membranes (Brown et al., 1990). Taken together, these observations suggest that the superficial fiber cells may play a much more active role in lens metabolism than previously suspected.

This study presents evidence that low molecular mass substances present in the lens epithelium do not have ready access to the fiber cell cytoplasm. If this picture is accurate, it will have important consequences for our understanding of the flow of material within the lens and the role of the lens epithelium in health and disease.

While this manuscript was in preparation, Prescott et al. (1991) published an account of intercellular dye communication in microinjected amphibian lenses. The present findings are consistent with those of Prescott et al., in that both groups were unable to detect dye transfer from the epithelium to the fiber cells. In contrast to the present study, however, dye transfer from the fibers to the epithelium was not detected in the amphibian lens.

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REFERENCES


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