Adrenergic Stimulation of Ciliary Process Epithelium
Causes Surface Membrane Internalization

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An ultrastructural change induced in the nonpigmented epithelium (NPE) of the ciliary processes by adrenergic stimulation in the albino rabbit was studied. Thirty min after topical treatment with 2% isoproterenol, an extensive intracellular membranous network, previously reported to be smooth endoplasmic reticulum, was revealed by electron microscopy. It was postulated that this network originated from the plasma membrane. Using cationized ferritin (CF) as an ultrastructural tracer, freshly isolated anterior segments were incubated in buffer containing $10^{-5}$ M isoproterenol and 0.2% CF. As early as 10 min, and for at least 30 min, the isoproterenol-treated NPE cells contained a membranous network that was morphologically similar to that which occurs in vivo. CF particles were present within the network, indicating that the membranous network had originated at the cell surface. This labeling was prevented by pretreatment with the $\beta$-adrenergic antagonist timolol maleate. In both treated and control ciliary processes, CF was present in the ciliary canals between the NPE and the underlying pigmented epithelium after 10 min incubation. This suggests that the NPE is able to transport CF from its basilar to apical surface. These experiments imply that the NPE is able to internalize rapidly large amounts of plasma membrane in response to adrenergic stimulation. This response may be part of the mechanism of adrenergic receptor desensitization, alteration of aqueous humor production, or another adrenergic response. Invest Ophthalmol Vis Sci 28:431-444, 1987

The major goal of medical treatment of glaucoma is reduction of elevated intraocular pressure. This goal is accomplished by reducing the production of aqueous humor by the ciliary processes and/or by increasing the outflow of aqueous humor from the eye. Among several classes of drugs that achieve these ends, adrenergic agents have proven clinically useful. Both epinephrine and timolol have therapeutically beneficial effects on aqueous humor dynamics. In addition, endogenous epinephrine may account for the circadian stimulation of aqueous formation in humans.1

The nonpigmented epithelium (NPE) of the ciliary processes participates in the production of aqueous humor by active transport mechanisms. The initial effects of neural and humoral stimulation on the NPE, particularly via adrenergic agents, have been extensively studied. $\beta$-adrenergic receptors are present, activated by epinephrine to stimulate cyclic AMP synthesis, and antagonized by timolol.2 However, little is known about the intracellular events following $\beta$-adrenergic stimulation. We have studied an ultrastructural change in the NPE of the albino rabbit induced by adrenergic stimulation.

Other investigators3,4 have described an increase in the volume of the smooth endoplasmic reticulum (SER) contained within NPE cells in the rabbit ciliary processes after topical instillation of isoproterenol. The increase of SER was most notable 30 min after drug instillation, and was prevented by pretreatment of the animal with a $\beta$-adrenergic antagonist. The volume of SER returned to control levels 1 hr after drug instillation.

Cells constantly shuttle plasma membrane and its constituents to and from the cell surface by way of intracellular compartments, such as Golgi, lysosomes, and smooth endoplasmic reticulum, in response to both extracellular stimuli and intracellular needs. Membrane recycling may occur at a high rate, for example, macrophages turn over an area equivalent to the entire cell surface every half hour.5 When internalized, however, the plasma membrane and its constituents have relatively long half-lives.

We reproduced the earlier findings of Ueno et al,3 confirming an increased volume of SER following topical isoproterenol, and reasoned that the rapid appearance of large amounts of intracellular membrane in the SER could not be accounted for by de novo membrane synthesis. We postulated that the increased volume of SER in the NPE following isoproterenol stimulation represented not de novo synthesis of SER, but...
rather plasma membrane that had been internalized from the basilar surface.

In electron microscopic studies, the use of cationized ferritin (CF), an ultrastructural probe which binds to anionic sites at the cell surface, allows visualization of the pathways for plasma membrane processing following internalization. We report observations on endocytosis and membrane traffic in the NPE following β-adrenergic stimulation, using CF as a membrane probe. Our results indicate that the increased SER does indeed represent internalized cell surface membrane in response to β-adrenergic stimulation.

Materials and Methods

In Vivo Topical Studies

50 μl of a 2% solution of l-isoproterenol was applied topically to one eye of adult albino rabbits. At 20, 30 and 40 min after drug instillation, the eyes were enucleated after sacrifice with an overdose of sodium pentobarbital. The contralateral eye in each animal was untreated. The globes were bisected at the equator and placed immediately in 2.5% glutaraldehyde in Millonig's Phosphate Buffer, pH 7.3. Following 30 min of fixation at room temperature, the partially fixed anterior segments were dissected to produce several pie-shaped pieces of iris-ciliary processes with an attached corneo-scleral rim. After a total of 3 hr of fixation at room temperature, the material was further processed for electron microscopy.

In Vitro Incubations

Adult albino rabbits were enucleated after sacrifice with an overdose of sodium pentobarbital. The globes were bisected at the equator and the vitreous and lens gently dissected free from the ciliary processes. Each anterior segment thus prepared was cut into four pie-shaped pieces. These pieces from paired eyes were pre-incubated under constant agitation at 37°C for 5 min in oxygenated Ringer's solution to which cationized ferritin (CF) had been added to produce a final concentration of 0.2%. L-isoproterenol was added to the incubating medium of tissue derived from one eye after 5 min of pre-incubation; tissue from the contralateral eye of the animal served as the control. Subsequent processing steps were identical to those described above.

Processing For Electron Microscopy

After aldehyde fixation, tissue pieces were postfixed overnight in 1% OsO₄ in Millonig's Phosphate Buffer, pH 7.3, dehydrated in ethanol and propylene oxide, and embedded in Epon/Araldite. Each experiment generated from four to six embedded specimens for each time point. Thin sections were cut from preselected areas (iridial, medial pars plicata and pars plicata) along the ciliary processes of at least two embedded specimens from each time point. For each condition and time point, from 10 to 20 well-preserved, cuboidal NPE cells with large areas of cytoplasm were examined and photographed.

Sections from the in vitro experiments were counterstained with 1% uranyl acetate and Reynold's lead citrate and examined in a Phillips 410 electron microscope. Specimens from the in vitro CF labeling experiments were examined unstained, because counterstaining was found to obscure the intracellular CF label.

Materials

Isoproterenol and cationized ferritin were from Sigma (St. Louis, MO). Timolol maleate was kindly provided by Merck, Sharp and Dohme (Rahway, NJ). Animals used in this study were cared for and treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Results

In Vivo Topical Studies

Twenty to thirty min following topical instillation of isoproterenol, there was an obvious increase in the volume of SER contained within the NPE cells of the treated eye. This response was most consistently observed at the medial portion of the pars plicata. SER-rich NPE cells were observed along the walls, crest, and crypts in this region. Responsive NPE cells containing SER-rich cytoplasm were also observed in other regions of ciliary processes of the treated eye, but at a lower frequency.

Within 10 min of drug application, the SER appeared in the perinuclear region towards the basilar aspect of the cell, and occupied much of the cytoplasm of stimulated cells (compare Fig. 1a, 1b). As the volume of SER increased, the Golgi apparatus, mitochondria, and lysosomes appeared unchanged. There was no noticeable change in rough endoplasmic reticulum or free
riboosomes, indicating that the increased volume of SER does not result from the stripping of ribosomes from the rough endoplasmic reticulum.

In both the control and isoproterenol-stimulated tissues, there were pinocytic vesicles at the NPE cell surface (data not shown).

**In Vitro Incubations With CF**

The ultrastructure of the ciliary processes after in vitro incubation with CF was well-preserved, and corresponded to that observed with in vivo control material. In the absence of counterstain, all structures were clearly visible. No abnormal changes in cell structure were observed for at least 35 min of total incubation. After longer incubation times, however, increasing vacuolization made micrographs difficult to interpret.

**Surface Labeling**

In both control and isoproterenol-treated tissue, incubation with CF for only 5 min resulted in distinct
labeling of the basolateral surface of the NPE cells. Although the density of CF labeling varied from region to region along individual ciliary processes, large areas along the ciliary processes were observed where CF particles were uniformly distributed, and appeared as a smooth and regular coating of electron-dense particles, following the contours of the plasma membrane (Fig. 2). The extracellular space between adjacent basalateral interdigitations was filled with CF particles.

In Vitro Stimulation of SER

Isoproterenol, present in the incubating medium at $10^{-5}$ M, caused an increased volume of SER that appeared morphologically the same as that seen in vivo following topical isoproterenol (Figs. 3, 5, 7). The SER occupied the perinuclear region after 10 min of incubation with isoproterenol, and increased in a time-dependent fashion, occupying large portions of cytoplasm...
of the NPE cell by 20 to 30 min. Because the tissue became increasingly vacuolated at 40 min and beyond, we were unable to determine what ultimately happened to the SER.

NPE cells not treated with isoproterenol contained a considerably smaller volume of SER; however, some SER was present in untreated specimens. Figures 4, 6, and 8 were selected to show areas of SER in untreated tissues. The sparse amount of SER in NPE cells of untreated tissues did not change during the course of the incubation in the absence of isoproterenol.

As shown in Figures 3b, 5b and 7b, the SER that resulted from isoproterenol stimulation contained CF particles. The CF particles appeared scattered randomly over the SER, and not clearly adherent in any pattern to the SER membranes. The preponderance of the CF labeled SER was found between the perinuclear region and the basal region of the NPE cells. The membrane

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**Fig. 3.** a. Ten min after incubation with $10^{-8}$ M isoproterenol and 0.2% CF, the NPE cell contains an extensive SER which occupies much of the cytoplasm of the cell. b. Higher magnification reveals CF particles contained within the SER (a X12,000), (b X31,000).
comprising this SER presumably originated at the cell surface during exposure to isoproterenol. The CF particles were associated with the membranes of the SER after 10 and 20 min of incubation with isoproterenol. At 30 min, there appeared to be a qualitatively lower density of particles scattered within the membranous network of SER.

Most NPE cells in untreated tissues contained few if any CF particles associated with the sparse SER. The cells in Figures 6 and 8 were selected to show SER in untreated NPE cells. As shown at high magnification (Figs. 4b, 6b, 8b), the SER remained unlabeled with CF.

Pre-Incubation With Timolol

Pre-incubation of the tissue with 10^{-5} M timolol maleate prevented the appearance of increased SER in response to the subsequent addition of 10^{-5} M isoproterenol. The morphologic appearances of untreated,
timolol treated, and timolol and isoproterenol treated tissues were identical (Figs. 9, 10). There was no CF labeling of the scant SER present. The mitochondrial swelling described by Hiramatsu et al.\textsuperscript{10} was not observed.

**Labeling of the Lysosomal Apparatus**

The elements of the lysosomal apparatus are endocytotic vacuoles, which have an electron-lucent content; light multi-vesiculated bodies (MVB), which are larger endocytotic vacuoles with some internalized vacuolar membrane profiles; dark MVB's, which contain a darker, electron-dense matrix; and dense bodies, similar in appearance to dark MVB's, but containing lipid droplets and myelin figures.

After 15 min of CF incubation, endocytotic vacuoles contained CF particles. Fusion of CF-labeled pinocytotic vesicles with the endocytotic vacuoles was commonly seen (Fig. 4a), suggesting discharge of the vesi-
cles' content into the vacuole with the resultant addition of membrane to the vacuole. CF particles were observed within both light and dark MVB's after further incubation; the CF particles appeared to be aligned with the internal membranous structure of the MVB's (Fig. 5a). Labeling of the Golgi apparatus was only rarely seen.

Labeling of the lysosomal apparatus was identical in control, isoproterenol-stimulated and timolol-treated tissues and is similar to that described in other tissues, ie, choroid plexus epithelium, cultured hepatocytes, and fibroblasts.

Labeling of the Ciliary Canals in Intercellular Space

In both the untreated and isoproterenol-stimulated tissues, we observed clusters of CF particles in the ciliary canals and the intercellular spaces between the NPE
and the underlying pigmented cells (Figs. 8b, 11). These clusters were present at all times examined, but were most prominent after incubations with CF of 20 min or longer. These CF particle clusters were often associated with vesicles opening into the intercellular space.

Many apical tight junctions of the NPE remained intact, suggesting that the barrier function in our in vitro preparations was not markedly compromised to CF. Because CF particles were not observed in the underlying stroma of the ciliary processes, appearance of clusters of CF particles in the intercellular space and the ciliary canals is unlikely to be the result of diffusion across the stroma.

**Discussion**

We have used cationized ferritin in vitro to demonstrate the internalization of cell membrane in NPE.
cells following stimulation with a $\beta$-adrenergic agonist. The nonpigmented epithelium of the ciliary processes, like many transporting epithelia studied to date with these techniques, has an elaborate system for the directed transport and recycling of plasma membrane. CF, which binds to the plasma membrane, is internalized at the basolateral surface of the NPE cells in vitro. We observe three different routes that are followed by the tracer: to the SER under conditions of $\beta$-adrenergic stimulation, to elements of the lysosomal compartments, and to the apical surface of the cells via transepithelial transport.

Membrane Internalization to the SER

In vivo and in vitro stimulation of the NPE with isoproterenol produces a rapid and obvious increase in the smooth endoplasmic reticulum of the NPE cells.
The increase in SER is prevented by the β-adrenergic antagonist timolol. Our labeling experiments indicate that this SER originates, at least in part, from the basolateral cell surface, and apparently contains an enormous surface area of internalized plasma membrane. Whether the increased volume of SER can be entirely accounted for by decreased basolateral plasma membrane area due to internalization will need detailed morphometric analysis with specific markers, and is beyond the scope of this work. At later time points in these experiments, the CF became scattered throughout the cell. This observation has been noted by other investigators, and limits the usefulness of CF in tracing subsequent steps in membrane traffic.

Investigators studying other secretory epithelia have postulated that all such cells share the ability to mod-
ulate rapidly their surface area, internalizing and externalizing plasma membrane as conditions require. The experiments described here suggest that the NPE cell is able to sequester a large portion of its overall membrane surface area in the SER in response to \( \beta \)-adrenergic stimulation. If membrane proteins, including pump sites, are sequestered along with membrane in the SER, one result would be to decrease the pumping ability of the NPE. This might contribute to the decreased aqueous humor inflow that has been observed in rabbits with adrenergic stimulation.\(^{14}\)

Down-regulation of the \( \beta \)-adrenergic receptor occurs
in response to β-adrenergic agonist stimulation by internalization of the receptor molecule, possible intracellular processing, and eventual return of the receptor to the cell surface. The phenomenon that we have observed occurs within minutes in response to a β-adrenergic agonist, and is specific in that a β-adrenergic antagonist blocks the response. Previously, β-adrenergic desensitization has been demonstrated in the iris-ciliary body of rabbits. The membrane internalization that we have observed might represent the morphologic appearance of the mechanism of β-adrenergic receptor desensitization.

Transport to the Lysosomal Compartment

This route for membrane traffic appears similar to that described in other cell types and employs internalization and merging of pinocytotic vesicles with endocytotic vacuoles to form MVB's. The role of the Golgi apparatus in subsequent steps of membrane traffic is controversial. Some investigators have demonstrated consistent CF-labeling of the Golgi apparatus, while others have failed to do so. In the NPE, the Golgi apparatus is rarely labeled, and may play a minor role in membrane processing.

There was no significant difference observed in either the overall morphologic appearance, or the frequency of large endocytotic vacuoles, MVB’s, or lysosomes when isoproterenol-stimulated and untreated tissues were compared. Membrane processing through the lysosomal compartment appears to be independent of β-adrenergic regulation.

Transepithelial Transport

NPE cells are capable of pinocytosis of extracellular CF and transepithelial transport of the resulting vesicles from the basolateral to apical surfaces of the cells. These vesicles merge with the apical surface and empty into the intercellular space between the nonpigmented and underlying pigmented cells. An intracellular route for CF is further implicated because this intercellular space does not communicate directly with the basolateral surface and passive diffusion is unlikely to account for the appearance of the label at the apical interface.

Van Deurs, et al described a similar finding in the choroid plexus epithelium of the rat. That tissue, which produces cerebrospinal fluid (CSF), is similar in both ultrastructural appearance and function to the NPE. The choroid plexus epithelium is known to clear the CSF of some inflammatory proteins and drugs, and these investigators have proposed that transepithelial transport, or “transcytosis”, may be one mechanism by which this occurs. Similarly, transepithelial transport by the NPE may be one of the mechanisms by which the posterior chamber is cleared of inflammatory proteins and subcellular debris.

When isoproterenol-stimulated and untreated tissues were compared for evidence of transepithelial transport, no significant differences were observed. Both tissues contained clusters of CF at the apical interface, and thus it appears that transepithelial transport is independent of β-adrenergic regulation.

Key words: adrenergic receptor, cationized ferritin, ciliary processes, nonpigmented epithelium, electron microscopy, isoproterenol, smooth endoplasmic reticulum

References


