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Ultracytochemistry of cholera-toxin binding sites in ciliary processes*

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Summary. Cholera toxin reduces the rate of formation of aqueous humor in concentrations (10^{-11}M) that do not disturb the morphology of the aqueous-humor forming epithelial cells of the ciliary processes of the rabbit eye. The search for an endogenous mediator of aqueous-humor formation comparable to cholera toxin in its mode of operation prompted us to map the distribution of cell surface receptors for cholera toxin in the ciliary processes of the eyes of rabbits. Cytochemical studies were carried out with the use of conjugates of cholera toxin to fluorescein isothiocyanate (CT-FITC) and to horseradish peroxidase (CT-HRP), and of the B subunit of cholera toxin to horseradish peroxidase (B-HRP). Multiple fluorescent CT-FITC binding sites were observed on the outer nonpigmented epithelial layer near the crests of the processes. Processes incubated with CT-HRP in vitro showed surface staining of 30–40 % of the nonpigmented epithelial cells. A prominent reaction product was observed along the basal and lateral plasma membranes of these cells. In vivo studies carried out after arterial infusion of B-HRP showed a reproducible dense reaction product between the apical surfaces of the pigmented epithelium (PE) and of the nonpigmented epithelium (NPE) facing each other. Aggregations of reaction product were observed with the electron microscope in the extracellular space between the apices of PE and NPE. The apical plasma membrane of the endothelium of the blood vessels near the crests of the ciliary processes was stained after either in vivo or in vitro exposure to peroxidase conjugates. These findings indicate that the cell-surface receptors which mediate the action of cholera toxin on aqueous humor formation are very likely localized in the apical plasma membranes of the epithelium of the ciliary processes.

Key words: Rabbit eye – Cholera toxin – Ciliary processes – Aqueous humor formation – Peroxidase conjugates

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Intraocular pressure is a result of the formation and drainage of aqueous humor within the eye. Aqueous humor is produced by the ciliary processes of the anterior portion of the ciliary body; the processes consist largely of a highly vascular stroma covered by a double layered epithelium. The layer adjacent to the stroma consists of pigmented cells; the layer facing the aqueous humor consists of nonpigmented cells.

The action of cholera toxin (CT) is mediated through its binding to receptors on the cell surface (Cuatrecasas 1973; Holmgren et al. 1973) and subsequent activation of adenyl cyclase on the inner membrane surface by the A_1 subunit of the toxin (Gill 1977; Moss and Vaughan 1979; Lai 1980). The mechanism by which the A subunit crosses the plasma membrane to reach the cytoplasm of cells binding the toxin is still under study (Wisnieski et al. 1981).

CT reduces intraocular pressure by decreasing net aqueous flow through the eye after arterial infusion of as little as 10^{-11} moles (Gregory et al. 1981; Sears et al. 1981). This new finding is consistent with an important role for adenyl cyclase in the maintenance of intraocular pressure by regulation of aqueous flow because CT stimulates adenyl cyclase in ciliary processes (Sears et al. 1981; Gregory et al. 1981), the tissue that produces aqueous humor. Studies of ocular adrenergic receptors supporting this concept have been reviewed elsewhere (Sears 1975). The purpose of the present work was to determine the cellular locus of action of CT on aqueous humor formation by looking for binding sites with fluorescent and peroxidase conjugates of CT.

Materials and methods

Male New Zealand white rabbits weighing 4 to 5 lbs were used. Cholera toxin (CT) was purchased from Schwarz/Mann Division, Becton, Dickinson and Co. (Orangeburg, N.Y.). The B subunit of CT was purchased from Calbiochem-Behring Co. (San Diego, Calif.).

Conjugation of cholera toxin to fluorescein isothiocyanate and ultrastructural study of conjugated cholera toxin-fluorescein isothiocyanate (CT-FITC)

CT-FITC was prepared by the method of Cebra and Goldstein (1965). For fluorescence microscopy small unfixed pieces of ciliary processes were incubated with CT-FITC for 30 min, 1 h, 2 h, or 3 h, and then washed in three changes of 0.85 % NaCl buffered with 10 mM sodium phosphate at pH 7.4 (PBS). Small ciliary pieces were frozen in liquid nitrogen, and sections were cut on a cryostat at $-20°$ C. The frozen sections were mounted on glass slides and air-dried. Sodium phosphate buffer (0.1 M, pH 7.4) was applied and coverslips were placed in position. Examination and photography were performed by means of a Leitz Ortholux microscope equipped with an Osram HBO/220-watt bulb. A BG-38 and a BG-12 filter (maximum 490 nm) were used as excitation filters and a K 510 (maximum 510 nm) was used as an analytic filter. The results were recorded on Kodak Ektachrome film (ASA 400). The usual exposure time was between 1 and 3 min. Three controls were prepared for each experiment. Tissue was not treated, incubated with fluorescein-labelled albumin, or preincubated with CT, washed with buffer, then incubated with CT-FITC.

Preparation of conjugates of cholera toxin to horseradish peroxidase (CT-HRP) or subunit B to horseradish peroxidase (B-HRP)

Coupling was performed with glutaraldehyde as a cross-linked agent (Avrameas and Ternynck 1971). In this method 20 mg of type VI horseradish peroxidase (HRP) (Sigma) were mixed with 400 µl of 1.25 % purified glutaraldehyde in 0.1 M sodium phosphate pH 6.8 for 18 h at 20° C in a light-tight container.

The excess glutaraldehyde was removed through chromatography on a Sephadex G-25 column equilibrated with PBS. The brown peroxidase fractions were pooled (3.0 ml) and 1.5 ml was used to dissolve the lyophilized contents of a vial containing 1 mg CT, or 0.6 ml of pooled fractions were used to dissolve the lyophilized contents of a vial containing 0.5 mg subunit B. This was followed by addition of as much saturated sodium bicarbonate, pH 9.5, as was required to produce pH 9.5. After 25 h at 4° C, 24 µl of 0.35 M lysine was added for an additional 2 h in order to bind unreacted glutaraldehyde. The reagent was exhaustively dialysed against PBS and stored at 4° C. These conjugates appear to retain specificity for receptors characteristic of the native toxin (Manuelidis and Manuelidis 1976; Joseph et al. 1979). Concentrations of B subunit or CT and HRP were calculated from absorbances at 403 and 280 nm. In our hands the usual molar ratio, HRP/CT or HRP/B was about 6:1.

Cytochemistry. HRP was detected by the method of Graham and Karnovsky (1966) by utilizing diaminobenzidine tetrahydrochloride (DAB) as substrate.

Ultrastructural studies for binding of CT-HRP or B-HRP. Excised pieces of ciliary body were used to study the binding of CT-HRP or B-HRP in vitro. All animals were killed by rapid injection of 5 ml flaxedil (20 mg/ml) into the ear vein. The eyes of all animals were quickly removed and divided into anterior and posterior parts under the dissecting microscope. The lens was gently removed mechanically and ciliary bodies were dissected into small pieces, each containing three or four processes. Some of the pieces of ciliary body were excised to include the iris and trabeculum. These were washed with cold (4° C) PBS and incubated with CT-HRP containing 500–700 µg/ml or B-HRP containing 320 µg/ml for 15, 30, or 60 min at 20° C. Three controls were prepared for each experiment. Tissue was not treated, incubated with unconjugated HRP, or first incubated with 200 µg CT/ml or 400 µg subunit B/ml, washed with buffer, then incubated with CT-HRP or B-HRP, respectively, as before. Dissected ciliary bodies were washed with cold PBS and fixed for 1 h with 1.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4, stained with DAB, postfixed in 1 % OsO_4, dehydrated, and embedded with Epon 812. Sections of ciliary bodies were stained with lead salts and examined in a Hitachi H-600 electron microscope.

In vivo binding of CT-HRP or B-HRP was studied by perfusion via the internal maxillary artery (Gregory et al. 1981; Sears et al. 1981) so that the blood supply to the eye was not compromised. Up to 500 µg CT and 1700 mg B subunit were used. Five to 30 min after infusion the animal was killed, the eyes were enucleated, and gentle dissection of the ciliary body was performed. Controls consisted of tissue from animals arterially infused with 1 ml saline, with 2 mg HRP, or with 1000 µg subunit B followed by infusion of 1000 µg B-HRP 5 min later.

Under ordinary conditions it would not be expected that molecules of the size of CT, 84 000 Daltons would pass the blood-aqueous barrier comprised of the tight junctions joining the NPE. However, a disrupted blood-aqueous barrier could allow access of CT to potential binding sites on the lateral interdigitating surfaces of the NPE of the ciliary body that are beyond the tight junctions of these cells. Indeed, with the very low doses of CT that can affect aqueous humor formation (2 µg, about 10^{-11} moles) the blood-aqueous barrier remains intact (Gregory et al. 1981; Sears et al. 1981). However, this small an amount of CT-HRP would not yield enough reaction product to permit localization of binding sites. Larger amounts of CT-HRP (500–1000 µg), sufficient to form a visible reaction product, would have caused disruption of the blood-aqueous barrier, and this could influence the distribution of CT-HRP, and therefore localization of binding sites. Since subunit B has no physiologic activity (intraocular pressure is unaffected, Mishima et al., unpublished results) but retains specific binding properties (Gill 1977; Moss and Vaughan 1979; Lai 1980) B-HRP could be used at concentrations high enough to form a reaction product visible with the electron microscope. Therefore, B-HRP was used to study binding sites under the normal conditions of an intact blood-aqueous barrier. As a control measure, disruption was induced by paracentesis of the anterior chamber in some eyes just before enucleation to check the effect of a disrupted blood-aqueous barrier on localization of binding sites.

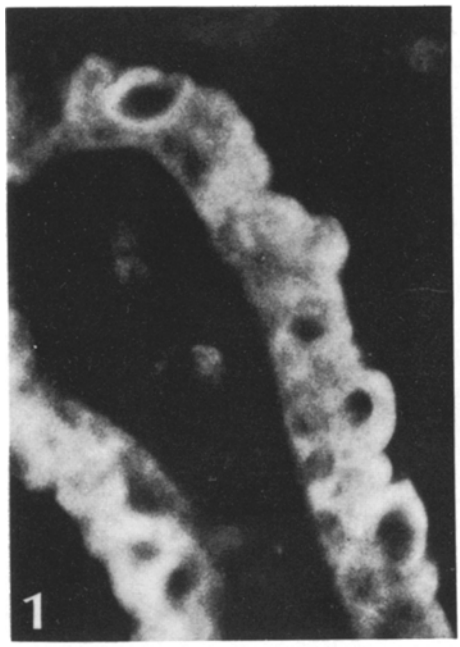
It should also be pointed out that in other work particularly designed to delineate the blood-aqueous barrier (Shiose 1970; Vegge et al. 1975), HRP was utilized as a protein tracer. One hundred to 500 mgs HRP, or about 100–500 µg/gm body weight, were usually required. Herein the receptor specificity of the B subunit permitted the use of tracer levels of HRP, 2 mg/animal, concentrations that were far below the level required for use as a label for the barrier. These low doses of HRP could not serve as a barrier label (see controls under Results).

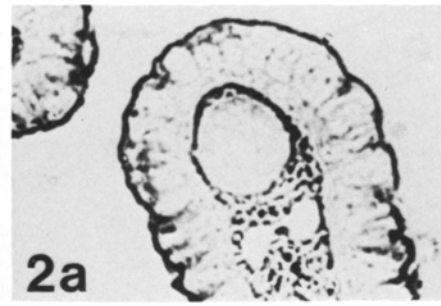
Results

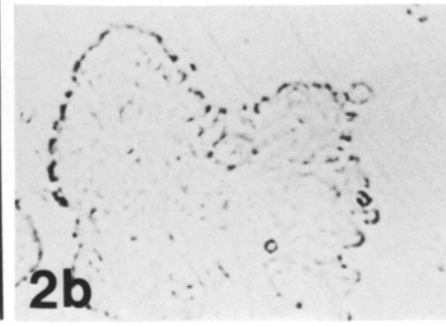
CT-FITC light microscopic study. No fluorescence was seen after 5 or 15 min of CT-FITC incubation. At 30 min a few fluorescent dots appeared on the outer epithelial layer near the crests of the ciliary processes. By 1 h, specific fluorescence became brighter and was seen along the length of the processes from crest to base; a bright fluorescence was also detected intracellularly in the NPE. Maximum staining occurred at the crests of the ciliary processes after 2 h exposure to CT-FITC (Fig. 1). A progressive reduction in fluorescence and in numbers of cells showing fluorescence occurred with longer incubation. Autofluorescent cells were rarely seen in untreated controls. In control experiments preincubation with unconjugated CT blocked any CT-FITC reaction.

Binding of CT-HRP and B-HRP in vitro. The reaction product of CT-HRP binding by ciliary processes was first seen under the light microscope after 15 min of incubation. The reaction product appeared on the crest of individual ciliary processes and seemed more intense in the anterior than in the posterior zone of the ciliary processes (Fig. 2a). On the crests of the processes a dense, "linear" reaction product appeared to occur on the surface of basal plasma membranes of the nonpigmented epithelial cells and sometimes between these cells. HRP staining intensified with longer incubation times and became more extensive as well. After 30 min of incubation with 500–750 μg/ml of CT-HRP, about 30–40% of nonpigmented epithelial cells showed surface HRP stain. The cytoplasm of some nonpigmented epithelial cells showed reaction product after 30 min or 60 min incubation. Some of the vascular endothelia within the stroma of the ciliary processes also showed intense HRP reactivity. In control experiments, preincubation of the tissue with unlabelled CT prior to incubation with CT-HRP effectively prevented the appearance of reaction product (Fig. 2b).

With electron microscopy, a prominent reaction product was observed along the basal plasma membranes of nonpigmented epithelial cells but not along the internal limiting membrane (Fig. 3). At higher magnifications, the reaction product seemed directly attached to the basal plasma membranes of nonpigmented epithelial cells. About the same frequency and intensity of HRP reaction product was noted in the region of the lateral interdigitations. The deposition of reaction product increased with incubation time. Reaction products were first seen in beaded form predominantly at the region of cellular interdigitations on the outside of the plasma membrane (Fig. 4). After incubation for 30 or 60 min reaction products accumulated so that they seemed to fill the lateral intercellular spaces between adjoining nonpigmented epithelial cells (Fig. 5). In contrast to the basal and lateral plasma membranes, the apical surfaces of nonpigmented cells showed no HRP reaction product after in vitro incubation. In addition, apparently intracellular vesicular structures, 80–150 nm in diameter, containing reaction product were observed in the basal cytoplasm of the nonpigmented epithelial cells. Dense reaction products were found on the apical plasma membrane of the endothelium of blood vessels, particularly those in the crests of the ciliary processes 30 or 60 min after the start of the incubation with CT-HRP (Fig. 6). Some nonspecific reaction product was found in the lumina of the ciliary blood vessels. Intense HRP reaction







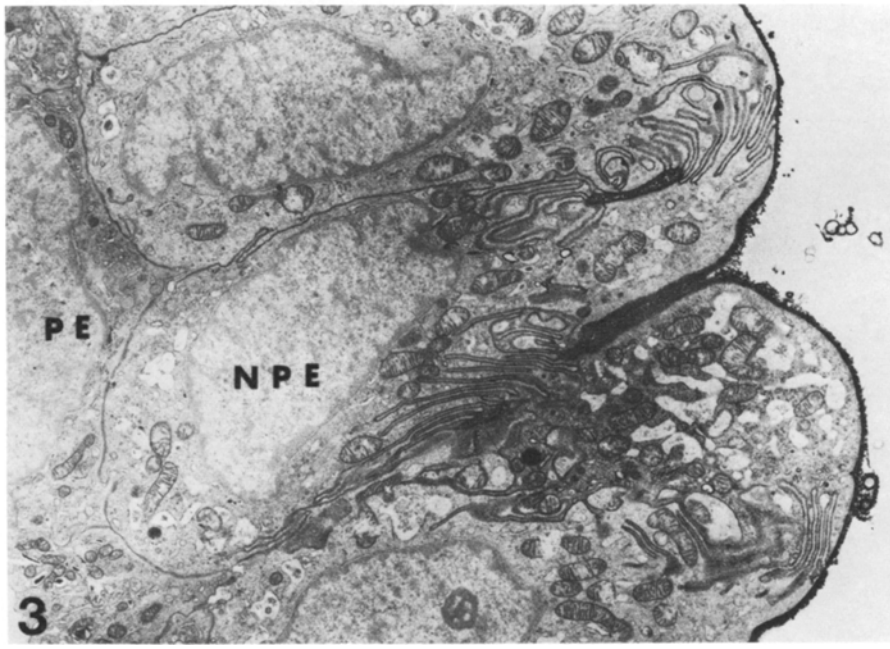


Fig. 1. Ciliary process 2 h after incubation with CT-FITC. Specific fluorescence concentrated along both epithelial layers. Stromal cells and vascular endothelia slightly fluorescent. ×160

Fig. 2a, b. Unstained thick sections of ciliary processes after treatment with CT-HRP or B-HRP. **a** Incubation with CT-HRP for 30 min. Note staining on surface and in intercellular spaces of epithelium at crest of ciliary process. Some vascular endothelia densely stained. **b** Incubation of tissue first with CT, then with CT-HRP. No reaction product. ×360

Fig. 3. Electron micrograph of part of ciliary process incubated with CT-HRP for 30 min. Reaction products at lateral interdigitations and spaces between adjoining NPE cells. *NPE* nonpigmented epithelium. *PE* pigmented epithelium. ×6,000

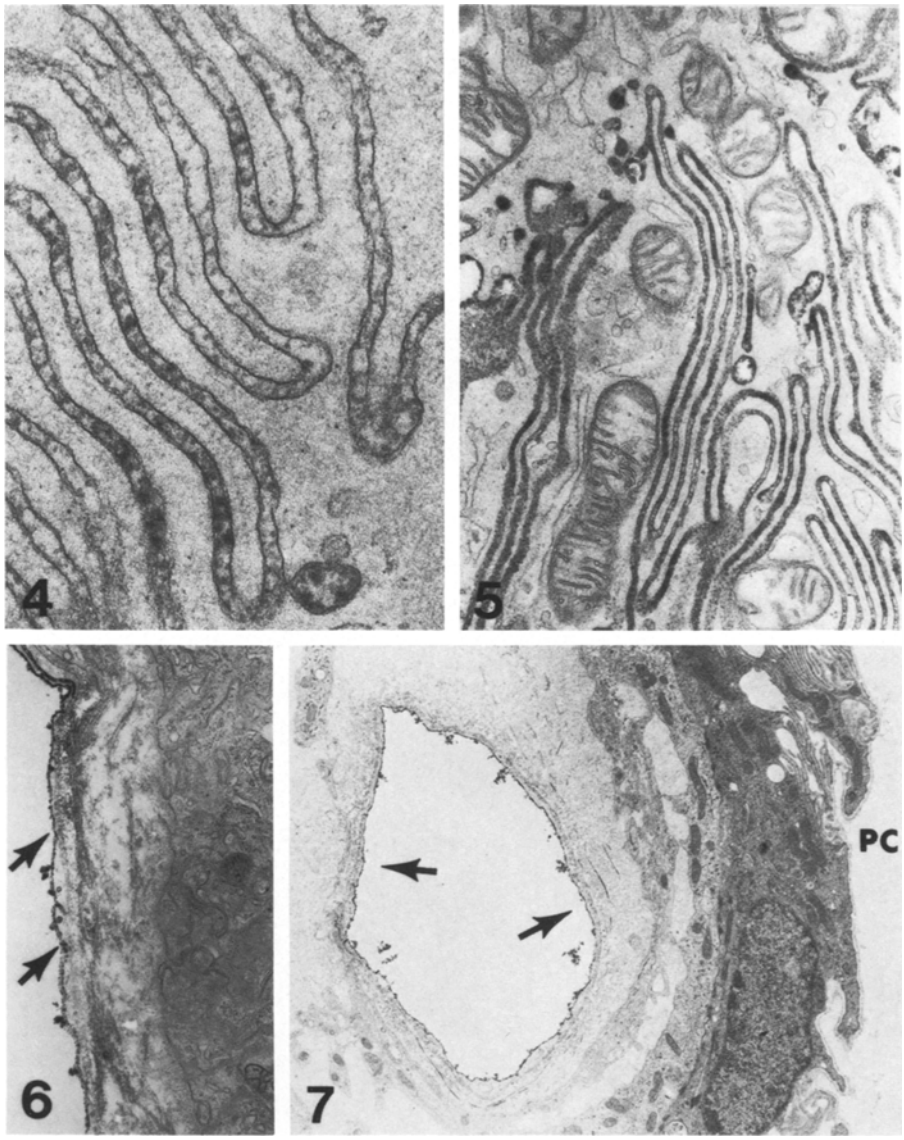
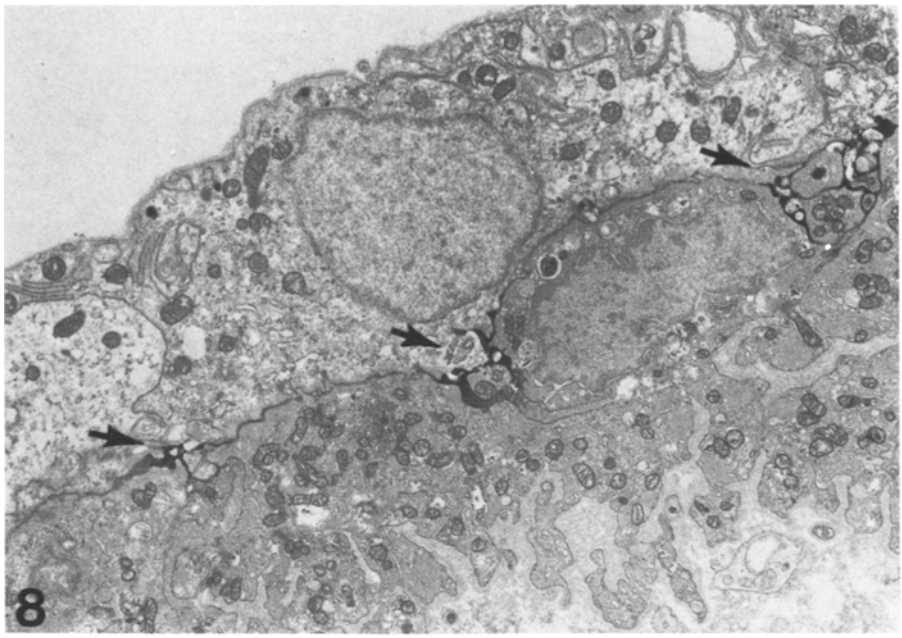


Fig. 4. Region of lateral interdigitations of NPE cells incubated with CT-HRP for 30 min. Beaded reaction products on outer surface of plasma membrane. ×60,000

Fig. 5. Lateral interdigitations of NPE cells incubated with CT-HRP for 30 min. Reaction products in intercellular spaces, 80–150 nm vesicles containing CT-HRP. ×17,000

Fig. 6. Electron micrography of part of a ciliary process incubated with CT-HRP. Reaction products on endothelia of ciliary blood vessels (*arrows*), but not on PE cytoplasmic membrane. *PE* pigmented epithelium. ×6,500

Fig. 7. Arterial infusion of CT-HRP: Electron micrograph of part of a ciliary process with stromal edema and enlargement of PE basal infoldings. In this instance reaction products (*arrows*) only at endothelia of ciliary blood vessels. Presumed slight diffusion of reaction product within stroma. *PC* posterior chamber. *ST* ciliary stroma. ×5,000



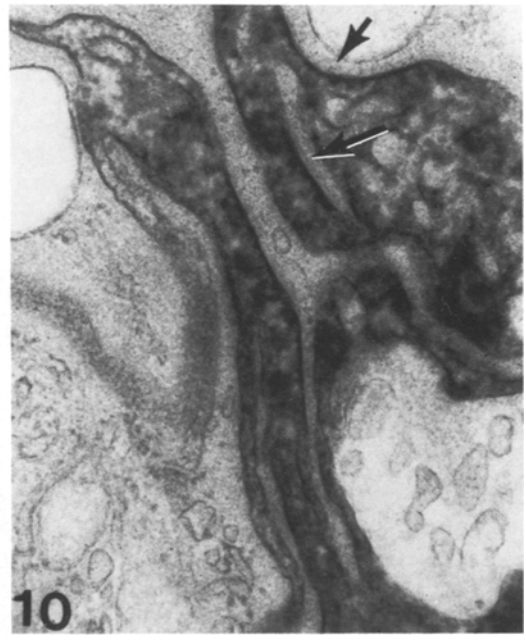


Fig. 8. Electron micrograph of part of ciliary process after arterial infusion with B-HRP. Reaction products (*arrows*) within apical extracellular spaces between PE and NPE cells. *PC* posterior chamber. ×6,000

Fig. 9. High magnification of apical extracellular spaces between PE and NPE cells. Specimen taken 5 min after arterial infusion of B-HRP. Reaction products filling these spaces. *PC* posterior chamber, *NPE* nonpigmented epithelium. *PE* pigmented epithelium. ×15,000

Fig. 10. Sequence as in Fig. 9. High magnification of part of extracellular space between PE and NPE cells. Reaction products clearly attached to PE or NPE cytoplasmic membranes (*arrows*). ×45,000

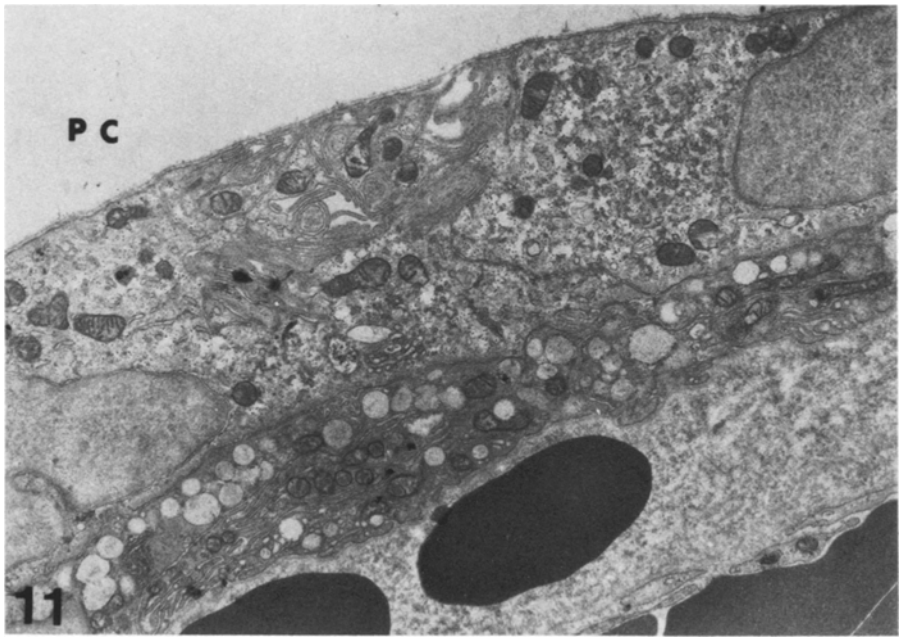


Fig. 11. Part of ciliary process after control infusion of 2 mg HRP. No reaction product. *PC* posterior chamber. ×6,500

products were never detected at the basal plasma membrane of the pigmented epithelial cells nor within intercellular spaces between adjacent pigmented epithelial cells (Fig. 6). No reaction product was observed in the iridocorneal angle or trabecular region.

Pieces of retinal and choroidal tissue were studied after in vitro incubations similar to those described for excised ciliary processes. No reaction product was observed on the photoreceptor cells or on the retinal pigmented epithelium, confirming the absence of reaction on the similarly constituted pigment epithelium of the ciliary process. Weak reaction products were detected in the nerve fiber layer, ganglion cell layer, and some of the choriocapillaris.

In vivo binding of CT-HRP and B-HRP. After arterial infusions of up to 500 μg CT-HRP a reaction product was observed on the endothelium of blood vessels near the crests of individual ciliary processes. The product appeared more intense in the anterior than the posterior zone of the processes. Reaction product appeared to diffuse within the stroma around the blood vessels although none was seen on either ciliary epithelial layer. The stroma of the processes showed an extraordinary edema without hemorrhage or inflammation, and both layers of the ciliary epithelia were intact and normal in appearance (Fig. 7).

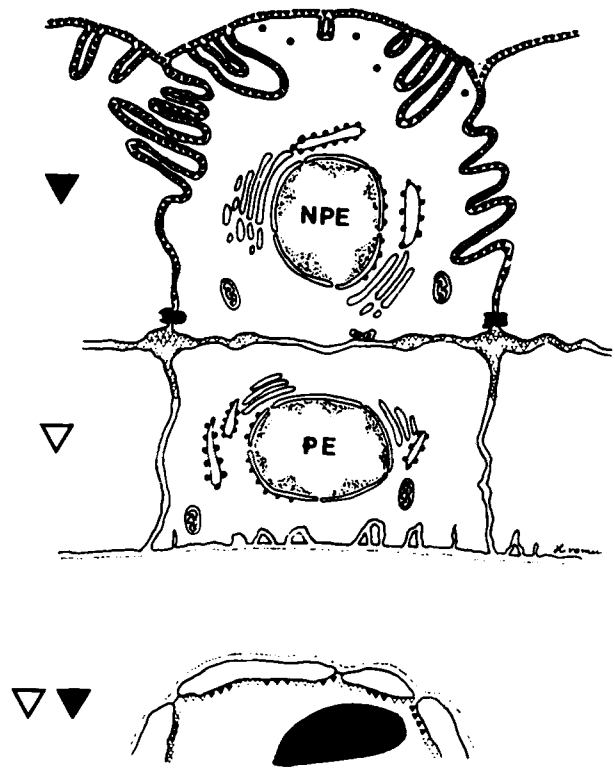
After infusion of B-HRP (1,700 μg) a characteristic dense dot-like reaction product occurred in the intercellular space between the apices of the nonpigmented and pigmented epithelial cells facing each other. Reaction product was also seen on the endothelia of some ciliary vessels. Only faint diffuse staining permeated the stroma around vessels near the crests of the processes and no significant stromal

edema was found. The most characteristic finding by electron microscopy was accumulation of intense reaction product in the extracellular space between the abutting of pigmented and nonpigmented epithelia and within the ciliary channels (Bairati and Orzalesi 1966) (Fig. 8). Higher mangification showed aggregates of reaction product, some of which appeared to be attached to apical plasma membranes (Figs. 9, 10). Occasionally the spaces between adjacent pigmented epithelial cells near their apices were filled with reaction product, but this deposition did not occur throughout the intercellular space from the base to the apex. Reaction product was never detected on the basal plasma membrane of the PE. Pinocytotic vesicles containing HRP reaction product were not observed in CT-HRP or B-HRP experiments done in vivo. Animals infused with 1 ml saline showed no morphological abnormalities. Control animals infused with 2 mg HRP alone or 1 mg subunit B followed by 1 mg B-HRP showed no reaction product (Fig. 11). Finally, the characteristic location of reaction product was unchanged after paracentesis, a procedure that induced only a mild disruption of the blood-aqueous barrier.

Discussion

Cholera toxin causes a remarkable reduction in the rate of aqueous-humor formation (Gregory et al. 1981; Sears et al. 1981). The aim of the present experiments was to find the site of action of CT by looking for specific binding sites. Our search for an endogenous mediator for the formation of aqueous humor that might have receptor sites similar to, or in common with, those for CT prompted an interest in the action of the latter delivered from the blood side as well as from the intraocular (vitreal) side of the ciliary epithelium. Furthermore, it turns out that the apical or "mucosal" side of one of the two cell layers that is presumed to be the important one for aqueous-humor formation, the NPE, points toward the blood. It was therefore of interest to see whether differences exist in binding sites found after delivery of the toxin via the blood, exposure to "mucosal" surface in the case of the NPE, versus serosal surface in the case of a delivery route by way of the vitreous, or after in vitro incubation. All of the plasma membranes of NPE cells contain CT binding sites, whereas only apical plasma membranes of PE cells show binding sites (Fig. 12). It is reasonable to conclude that the localization of reaction product after arterial infusion yields information on the specificity of CT binding sites and does not simply reflect the limitation imposed by the blood-aqueous barrier. The most prominent CT binding sites after arterial infusion of CT appeared at the crests of the anteriorly located ciliary processes. Whether this crest preference reflects differences in regional blood flow, and therefore in amounts of agonist delivered, is not possible to resolve at this time.

Although the molecular structure of the CT receptor has been viewed from several standpoints (Cuatrecasas 1973; Holmgren et al. 1973; Craig and Cuatrecasas 1975; Donta 1976; Kanfer et al. 1976; King et al. 1976; Morita et al. 1980), there is general agreement on certain characteristic physiologic events which occur after binding of CT to the cell surface. One of these is a lag period before activation of adenyl cyclase (Carpenter et al. 1968). This lag period is thought to represent the time required for the toxin to be transported from the binding site on



12

Fig. 12. Diagram of distribution of CT or subunit B binding sites on ciliary processes. *Closed triangles*, binding sites after in vitro incubation. *Open triangles*, binding sites after in vivo infusions

the plasma membrane to its intracellular site of action (Parkinson et al. 1972). Anatomic relationships may or may not contribute to the lag period. For example, in the case of the intestine, CT applied to the mucosal surface is very effective in activating adenyl cyclase (Peterson et al. 1972). It is less effective applied from the serosal side (Field et al. 1972; Sheerin and Field 1977) or delivered via the blood (Pierce et al. 1972). It is thought that the adenyl cyclase of intestinal epithelium is not concentrated in the brush border (Parkinson et al. 1972). It may well be concentrated on the blood front of the cell. In other words, even though binding occurs at the mucosal surface, adenyl cyclase activation may occur elsewhere in the cell.

In the case of the eye, access via the blood to other than the apical (brush) borders of the NPE is prevented for large molecules by the tight junctions of the NPE (Vegge et al. 1975). CT arriving via the blood could not be bound beyond the brush or apical border of the NPE. In vitro, however, or after intravitreal injection, the toxin has access to and is bound by the basolateral cell surfaces of the NPE. Low doses of CT decrease intraocular pressure and aqueous humor formation from either "mucosal" (blood) or "serosal" (vitreous) surfaces (Gregory et al. 1981; Sears

et al. 1981). In either case, an interval occurs that probably reflects the time required for the toxin; i.e., its active A_1 subunit, to be transported from the binding site to the intracellular locus of action. Both in vitro and in vivo experiments reported here indicate that there is a very distinct preference of CT for the NPE membranes as compared to the PE membranes suggesting that activation of adenyl cyclase mediated secretion occurs through the NPE cell layer. This conclusion is supported by Tsukahara and Maezawa (1978) who reported that adenyl cyclase activity is distributed almost exclusively on the plasma membranes of the NPE, and not of the PE. These anatomic findings, taken together with the observation of reaction product on the apical border of the NPE in ciliary processes excised and studied after blood stream delivery of toxin, strongly suggest that binding of CT to this border is followed by intracellular activation of adenyl cyclase in this or other membranes of the NPE to produce a decrease in net aqueous flow. Whether or not PE membranes participate at all in this process is a problem that has not yet been definitely solved, but appears unlikely.

Vascular participation in the events occurring after CT administration is an issue that must be discussed. After both in vitro incubation and arterial infusion with peroxidase conjugates, a reaction product was found on vascular endothelia in varying degrees. Furthermore, after intra-arterial perfusion of CT (Gregory et al. 1981; Sears et al. 1981) a prominent swelling of the ciliary stroma occurred associated with a doubling of ciliary-body blood flow. In other tissues, especially skin, injected or perfused with CT (Keusch et al. 1967; Kennedy et al. 1972) increased capillary permeability, vasodilation, and mucosal edema can occur. Therefore, it is important to consider whether the vascular binding sites demonstrated here may reflect hemodynamic factors responsible for the decrease in net aqueous-humor flow previously noted. Cedgard et al. (1978) reported a 50% increase in intestinal blood flow after exposure of the cat's small intestine to CT. For this and other reasons these authors suggested that increased capillary filtration pressure may play a role in the pathogenesis of cholera. Studies in rat intestine by Peterson et al. (1972) and in rabbit intestine by Yardley and Brown (1973) utilizing HRP as a marker, demonstrated that the epithelial layers were not disrupted after exposure to CT and that there was no stromal leakage of HRP. DiBona et al. (1974) indicated that secretion induced by CT was unrelated to increased hydrostatic pressure but was more consistent with a change in the ion transport properties of the epithelial cells. Recently Goerg et al. (1980) reported that after exposure of the luminal cell surface to CT epithelial continuity in the rat colon was preserved and epithelial secretion was enhanced. Ordinarily the ciliary stroma contains a protein concentration approximately 75% of that of the plasma (Bill 1975). This occurrence undoubtedly reflects the fenestration of the ciliary capillaries. The effect of this high concentration of protein in the stroma would induce a very slight backward flow of water from the posterior chamber into the ciliary processes. At these high protein levels increased capillary permeability might be expected to increase further the stromal concentration of protein only very slightly. Thus, it is unlikely that hemodynamic changes, such as increased blood flow and vasodilation seen after CT, can cause the decrease in intraocular pressure and net aqueous flow. The latter event, supported by the anatomic observations reported here, is best explained by binding of the toxin to the surface of the

nonpigmented epithelial cell layer of the ciliary processes with subsequent direct effects on cell metabolism.

For completeness, brief mention should be made of other related issues the solution of which are beyond the scope of this paper. It is possible that the same or different ciliary epithelial cells have "absorptive" as well as secretory function. Evidence to support this hypothesis is sparse (Pappas and Smelser 1959) and the last attempt to characterize the physiologic features of the "absorptive" process was in 1973 (Bárány). Physiologic studies indicated a decrease in net flow (Gregory et al. 1981; Sears et al. 1981) but whether CT is affecting secretion or absorption or both is a question not yet addressed. Second, it is possible that the reduced net aqueous flow occurring after CT is not entirely linked to cyclic AMP. A study specifically linking aqueous flow or changes in ion transport across the ciliary epithelia to changes in cyclic AMP has not yet been done. From the dose-response curves, the characteristic lag period, and what is known about the mechanism of action of CT, it is likely that adenyl cyclase activation is very important in mediating most of the response. Finally, the issue of whether there is a direct transfer of cyclic AMP from adenyl cyclase to either a protein kinase or a phosphoprotein phosphatase that is in turn linked to a membrane phosphoprotein is unclear for this system. Further study is required.

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