Studies on the cornea

IV. Some effects of ouabain on pinocytosis and stromal thickness in the rabbit cornea

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This study is concerned with the morphological manifestations of ouabain treatment as reflected in corneal thickness and pinocytosis. Administration of ouabain in vivo or in vitro leads to corneal swelling and a marked reduction in the number of pinocytotic vesicles in the endothelium. A comparison of ouabain-treated, cold-inhibited, and neonatal corneas, as well as consideration of electron histochemical studies on ATPase distribution in the cornea, suggests that a common mechanism may underlie the morphological changes and that the pinocytotic transport system in the corneal endothelium may be dependent on an electrolyte transport system for proper function.

Previous reports from our laboratories have described the process of pinocytosis in the endothelium of the rabbit and frog cornea,1-4 as well as the existence and function of an "Na+ pump" in the adult rabbit corneal epithelium.5,6 Preliminary investigations7 have shown that the cardiac glycoside, ouabain, reversibly inhibits epithelial active Na+ transport. In addition, the demonstration of an ATPase in the corneal endothelium8-10 and reinterpretation of the earlier electron micrographs of the corneal endothelium1-3 strongly suggest that an active electrolyte transport system occurs in this structure.

The present investigation was undertaken to determine whether the pinocytotic process in the corneal endothelium could be altered or abolished under the influence of ouabain, administered in vivo or in vitro.

Materials and methods

Adult rabbit corneas were exposed, either in vivo or in vitro, to ouabain in concentrations ranging from 10^{-4} to 10^{-6}M.

1. In vivo exposures were made in rabbits minimally anesthetized with Nembutal or ether. Ouabain was administered, either by intravenous injection of 0.4 ml. per kilogram of a solution containing 0.25 mg. of ouabain per milliliter, or by intra-ocular injections of 0.2 ml. of a 5 x 10^{-5}M ouabain solution. Following injection of ouabain by either route, measurements of corneal thickness were made with a corneal pachometer, as described by Maurice and Giardini.13 When the cornea had swelled to a thickness which remained constant for 10 to 20 minutes, an injection of
approximately 0.2 ml. of a 25 per cent solution of ThO₂ (Thorotrast, Testagar & Co., Inc., Detroit, Michigan), or of a 25 per cent solution of saccharated iron oxide was made into the anterior and posterior chambers of the eye and left for 15 to 30 minutes. In animals given ouabain intraocularly, the ouabain was usually given in the left eye with the right eye serving as a saline-injected control.

2. In vitro studies were carried out in the chamber previously described by addition of a 0.25 per cent ouabain solution to the perfusing medium to achieve a final concentration of 10⁻¹, 10⁻², or 10⁻³ M. Corneas were allowed to develop a potential in this medium for approximately one hour, at which time 0.6 ml. of a 25 per cent solution of ThO₂ was added to the solution bathing the endothelial side. Thirty to sixty minutes later the corneas were removed from the chamber, fixed, and processed for electron microscopy as described below.

All corneas were fixed either in 1 per cent OsO₄ in Sorenson's phosphate buffer at pH 8.2 or in 6 per cent glutaraldehyde in the same buffer followed, after appropriate buffer rinses, by fixation in 1 per cent OsO₄ in the same buffer. In vitro corneas were fixed only by the latter procedure. Corneas were dehydrated in a graded series of ethanol or acetone and embedded in Epon 812. Those corneas dehydrated in acetone were stained during dehydration by addition of KMnO₄ to one of the 100 per cent acetone changes, according to the method of Parsons. Sections were cut on a Porter-Blum MT-1 microtome by means of glass or diamond knives and stained with uranyl acetate and lead citrate before examination in an RCA EMU 3-C electron microscope.

Results

The administration of ouabain either intravenously or intraocularly produces at 10 to 120 minutes an increase in corneal thickness of 9 to 24 per cent with an average of approximately 16 per cent (see Table I). Contralateral control corneas for intraocular injection of ouabain showed no significant change in corneal thickness during the experimental period (Table I).

Examination of over 100 ouabain-treated corneas exposed to ThO₂ for 10 to 40 minutes in vivo or in vitro shows consistent morphological changes. The ouabain-treated cornea has a paucity of small vesicles in the apical cytoplasm of the endothelium (Fig. 1). Although the marker is found adsorbed on the extraneous coat of the apical plasma membrane and in small amounts in the intercellular space, that found within membrane-bounded compartments in the cytoplasm is usually in large, densely filled vacuoles (Fig. 2).

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*Cataract produced by injection.
†Ether anesthesia.

Table I. Sample corneal measurements for ouabain administered intravenously or intra-aqueously and for saline controls
Fig. 1. Portion of two endothelial cells from a cornea exposed to ThO$_2$ in the anterior chamber after intravenous administration of ouabain. A small amount of ThO$_2$ is adsorbed on the apical surface and some is seen in the intercellular space (IC) but only a single vesicle (V) containing ThO$_2$ is seen in the apical cytoplasm of each cell. Some marker has reached Descemet's membrane (D). This pattern closely resembles the cold-treated cornea (Fig. 3) and should be compared with the normal cornea (Fig. 4). N, nucleus. (x32,000.)

The small number of pinocytotic vesicles containing the marker (1 to 2 per electron microscopic field) closely approximates the number found when corneas are exposed to ThO$_2$ at 0 to 4°C. In the cooled cornea (Fig. 3) the marker is also found at the apical surface as well as in the intercellular space, but few marker-filled vesicles are found in the apical cytoplasm. It was estimated that cooling reduced the number of vesicles by 70 to 80 per cent. The effect of ouabain is at least as great.

The normal cornea (Fig. 4), exposed to ThO$_2$, shows uptake of the marker into numerous small pinocytotic vesicles in the apical cytoplasm of the endothelium, particularly in the region adjacent to the apical complex. Previous studies have shown that the marker is then carried around the apical complex in these vesicles. Fusion of the
Effects of ouabain on pinocytosis in rabbit cornea

Fig. 2. Portion of the apical cytoplasm of an endothelial cell from a cornea treated in the same way as Fig. 1. A portion of the nucleus (N) and a prominent Golgi zone (G) are shown. Large, densely filled vacuoles (VA) appear associated with the Golgi zone. The smaller vacuoles surrounding one of these, as well as poorly defined internal structure in the vacuole, suggest formation by fusion of smaller elements. (x38,700.)

Discussion

While there have been many studies of the biochemical and pharmacological effects of the cardiac glycosides, particularly ouabain, on electrolyte transport and cation-activated ATPase activity, there previously has not been an investigation of its effect on pinocytosis. The cornea, as one of the few tissues in which pinocytosis has been demonstrated to be a true transport process, provides a unique site in which to study these effects.

The initial step in pinocytosis is adsorption of material or solute at the cell surface. Numerous studies have shown that this step is reversible until changes occur in the physical-chemical state of the cell membrane which lead to the formation of a vesicle and the concomitant internalization of the adsorbed solute. The adsorptive and vesicle-forming steps have been demonstrated in the cornea and many other sites under widely varying conditions.

However, the question of what produces the apparently directed movement of the vesicles from the apical to the lateral membrane of the corneal endothelial cell has remained one of the puzzling aspects of pinocytosis as a transport mechanism. Interpretation of the present results, in conjunction with the results on corneas studied...

*See especially bibliography of Brandt’s paper.*
Fig. 3. Portion of two endothelial cells from a cornea exposed to ThO₄ after cooling to 0°C in vitro. Marker is adsorbed at the apical surface and some is found in the intercellular space (IC) but, again, there is a paucity of small vesicles (V) in the apical cytoplasm. N, nucleus; D, Descemet's membrane. (×33,300.)

at 0°C, and recent work on ATPase localization in the cornea suggests an answer to this question based on a possible relationship between electrolyte transport and pinocytosis. This relationship between electrolyte transport and pinocytosis would involve a system having the following characteristics:

1. An electrolyte pump located in the lateral margin of the endothelial cell would establish an intracellular fluid current...
Effects of ouabain on pinocytosis in rabbit cornea

Fig. 4. Portion of two endothelial cells from a normal cornea exposed to ThO₂ in vitro. A large amount of marker is adsorbed at the surface and marker fills the intercellular space (IC). Of most significance, however, is the large population of vesicles (V) in the apical cytoplasm, many of which appear to be passing around the apical junctional complex (J) prior to fusion with the lateral membrane. (x33,300.)

which would carry vesicles toward the lateral membrane. Evidence for the location of such a pump at this site comes from several sources. Chemical and histochemical evidence indicates that there is an ATPase located in the corneal endothelium. Electron histochemical studies demonstrate that a relatively substrate-specific ATPase is located on the lateral cell membranes of the endothelial cells and that pinocytic vesicles in these cells are negative for ATPase activity. Other sites of electrolyte transport such as the frog skin, gallbladder, and colon exhibit similar localization of ATPase activity. The cornea of the newborn rabbit does not exhibit pinocytotic activity until approximately 6 days after birth. Electron histochemical studies show that ATPase activity on the lateral membranes of the corneal endothelial cells is first exhibited at the same age. Both of these changes are concomitant with the clearing of the cornea. A pump located on the lateral membrane would, according to the theoretical construct of Curran, probably use the intercellular space as the intermediate fluid compartment in the three-compartment transport system. Confirmation of the Curran model has recently come from the work of Kaye, Lane, Wheeler, and Whitlock on the gallbladder epithelium, a system which exhibits all the morphological, histochemical, and physiological characteristics of the pump described above.

It must be understood, however, that the
pump hypothesized for the corneal endothelium is a "neutral pump," i.e., one which moves specific cations and anions and is dependent on both for its function. This type of pump is found in the ileum and the gallbladder, in both of which a nearly isotonic solution of NaCl is pumped by the epithelium, resulting in a bulk flow of solvent across the epithelium. The "pumps" in the ileum and gallbladder remove NaCl from the interior of the cell at the lateral surface, creating a hyperosmotic compartment in the intercellular space into which water moves from the cell. Cellular electrolyte and water balance is maintained by diffusion of NaCl and water into the cell at the apical surface. Therefore, it is likely that this bulk flow of solvent which accompanies movement of electrolytes carries small vesicles with it toward the lateral cell margin.

In addition, in the gallbladder, the size of the intercellular space varies directly with electrolyte-dependent fluid transport. Swelling of the intercellular space of the corneal endothelium, which has been noted previously by us and by Speakman, again suggests that a similar system exists in the corneal endothelium. Furthermore, movement of colloidal particles in the intercellular space is unidirectional into Descemet's membrane where they distribute as if moving in a fluid current.

Such a pump should be inhibited by ouabain and cold. Both cold and ouabain treatment (Table I) produce a reversible corneal edema. Donn and his co-workers have shown that both treatments inhibit active sodium transport in the corneal epithelium. This transport is presumed to be dependent upon an ATPase activity which has been demonstrated in the corneal endothelium and epithelium. In vitro chemical studies of the endothelial ATPase have demonstrated ouabain sensitivity. Recent studies by Tice and Engle and Bonting and Caravaggio have demonstrated that the activity of the cation-activated ATPases of the sarcoplasmic reticulum, squid axon,
frog skin, toad bladder, and other tissues is
very sensitive to temperature changes.

3. Inhibition of this pump would stop
the directed flow of fluid and vesicles with-
in the cell and would also stop the directed
diffusion of electrolytes to and across the
apical plasma membrane of the cell. The
static state thus produced at the apical
plasma membrane would tend to reduce
or eliminate vesicle formation. In corneas
studied at 0° C.3 or treated with ouabain
(Figs. 1 and 5) there is a marked reduc-
tion in the number of vesicles found in the
apical cytoplasm. The rows of vesicles
lateral to the terminal bar zone which are
seen in the normal cornea13 (Fig. 4) are
lacking under these conditions.

4. The loss of a directed intracellular
flow could lead to random collisions of
marker-filled vesicles and their subsequent
fusion into densely filled vacuoles within
the cell. Large vacuoles containing colloidal
markers are found in the ouabain-treated
cornes (Fig. 2), as well as in the neo-
natal cornea prior to the start of normal
pinocytotic activity.31

It would appear, therefore, from the
similar morphological findings in ouabain-
treated, cold-inhibited, and neonatal cor-
neas, as well as histochemical and physio-
logical data, that some relationship exists
between electrolyte transport and pino-
cytosis. Further investigation is necessary,
however, before the conclusion can be
drawn that an electrolyte pump is actually
the driving force for pinocytosis in the
corneal endothelium.

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