Studies on the cornea. V. Electron microscopic localization of adenosine triphosphatase activity in the rabbit cornea in relation to transport

Gordon I. Kaye* and Lois W. Tice**

The distribution of ATPase activity in adult and 7-day-old rabbit corneas has been studied by electron microscopic-histochemical techniques. In the corneal endothelium, the lead phosphate end product of the ATPase reaction is localized at the lateral margins of the cells and in the intercellular spaces. This activity is first demonstrable at 7 days. Pinocytotic vesicles, even those clearly containing ThO₅ marker, show no nucleotide phosphatase activity. Epithelial ATPase is less fixation resistant than endothelial ATPase, but, after short glutaraldehyde fixation, end product is localized in the intercellular spaces.

The frequent finding of histochemically demonstrable nucleoside phosphatase activity in pinocytotic vesicles has led some investigators to suggest that this activity may be involved in transport by these vesicles. The relation between this activity and vesicular function has remained obscure, however, primarily because most of the morphologically recognizable pinocytotic vesicles studied have not yet been shown to be physiologically active in transport. In the corneal endothelium, pinocytosis has been demonstrated to be a true transport process, and the morphological aspects of this process have been intensively studied. In the experiments reported here, investigation of the histochemical distribution of phosphatases in corneal endothelium was carried out in the hope that the results might clarify the relationships among pinocytosis, phosphatase activity, and transport.

Materials and methods

Corneas of adult and 6- and 7-day-old rabbits were fixed for 5, 30, and 120 minutes in 1 to 6 per cent glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, with or without addition of 0.2M sucrose. Other adult corneas were fixed for 2 hours in...
4 per cent glyoxal in 0.2M cacodylate buffer, pH 6.5, containing 0.4M sucrose and for one hour in 0.5 per cent hydroxyadipaldehyde in 0.1M cacodylate buffer, pH 7.0, also containing 0.4M sucrose.

After appropriate buffer rinses, corneal slices, or segments were incubated in the Wachstein-Meisel medium12 for the demonstration of ATPase activity.

In control experiments, corneas were incubated in the medium from which substrate was omitted, or in which equimolar amounts of ADP, AMP, CTP, IDP, or β-glycero phosphate were substituted for the ATP normally used as substrate. The reaction was followed in the light microscope by periodically examining thin slices of cornea which had been treated with dilute ammonium sulfide to convert precipitated lead phosphate to black lead sulfide; when a visible reaction was noted (10 to 30 minutes), incubation of the material to be used for electron microscopy was stopped.

Approximately half of the adult and newborn corneas studied were exposed to ThO2 for 10 to 30 minutes prior to fixation by injection of the marker into the anterior chamber. In addition, some ThO2 injected corneas either were not incubated in the ATPase medium or were used as substrate-free controls.

At the end of the incubation, corneas were rinsed thoroughly in buffer and post-fixed in buffered 1 to 2 per cent OsO4 for 1 to 2 hours. Dehydration and embedding in Epon 812 were carried out as described previously. Sections were cut on a Porter-Blum microtome using diamond knives and were routinely stained with 1 per cent uranyl acetate in 50 per cent alcohol and in lead citrate prior to examination in an RCA EMU 3-G electron microscope.

Results

Corneas fixed in glutaraldehyde and incubated in the histochemical medium containing ATP as substrate showed precipitated lead phosphate to black lead sulfide. Thorotrast, Testagor and Co., Detroit, Mich.

Fig. 1. In this portion of rabbit corneal endothelium reacted for ATPase activity in the Wachstein-Meisel medium,12 lead phosphate end product is localized in the intercellular space.

(Original magnification ×32,000.)
Fig. 2. For legend see opposite page.

Fig. 3. For legend see opposite page.
Fig. 2. In a ThO$_2$ control, incubated in substrate-free medium, only ThO$_2$ is in the endothelium. The marker is adsorbed at the apical surface and is in vesicles in the apical cytoplasm. The fusion of vesicles with the lateral cell membrane (arrows) is particularly well demonstrated in the glutaraldehyde-fixed material. (Original magnification x34,000.)

Fig. 3. In this corneal endothelium which was reacted for ATPase activity after injection of ThO$_2$ into the anterior chamber, both ThO$_2$ and lead phosphate are found in the intercellular space. Vesicles and vacuoles in the apical cytoplasm which contain ThO$_2$ have no lead phosphate end product (arrows). (Original magnification x38,000.)

Fig. 4. In this unstained section of a cornea treated in the same way as that shown in Fig. 3, the denser particles of ThO$_2$ are discernible within the lead phosphate deposits in the intercellular space. Whereas vesicles free in the cytoplasm (V) have no lead phosphate associated with them, two vesicles which have fused with the lateral plasma membrane (arrows) show an ATPase reaction. (Original magnification x32,000.)

The precipitated final product, lead phosphate, in the corneal endothelium (Fig. 1) closely related to the lateral plasma membranes of the endothelial cells and in the intercellular spaces. Pinocytotic vesicles in the apical cytoplasm contained no precipitated lead phosphate (Figs. 1, 3, 4).

Corneas which had been exposed to ThO$_2$ in the anterior chamber for 30 minutes prior to fixation showed uptake of the marker into vesicles. These specimens also demonstrated the apparent movement of marker-filled vesicles around the apical complex, their fusion with the lateral cell membrane (Fig. 2), and the extrusion of their contents into the intercellular space, a sequence of processes previously reported. The fusion of vesicles with the
Figs. 6-10. In these portions of corneal endothelium incubated with ADP, AMP, CTP, IDP, and β-glycerophosphate, respectively, slight reaction, or no reaction at all, is seen in the intercellular space. (Figs. 6, 8, original magnification ×17,100. Figs. 7, 9, 10, original magnification ×32,700.)

Fig. 5. This portion of corneal endothelium of a 7-day-old rabbit shows a weak positive reaction for ATPase in the intercellular space. This is the earliest stage at which this reaction has been found. A vesicle (V) contains ThO₂ which is first taken up by the corneal endothelial cells at 4 to 8 days. (Original magnification ×39,700.)

Lateral membrane is particularly well demonstrated in all glutaraldehyde-fixed corneas (Fig. 2, arrows).

Corneas exposed to ThO₂ and subsequently incubated in the ATPase medium also showed marker in pinocytotic vesicles and in the intercellular space, indicating transport by the vesicles. In such doubly marked preparations (ThO₂ and lead phosphate), the results obtained with each marker were equivalent to those obtained with the markers used separately. Despite the unequivocal identification of pinocytotic vesicles by clearly visible ThO₂ particles within them (arrows, Fig. 3), no lead phosphate was seen associated with the vesicles but was again restricted to the intercellular space (Figs. 3 and 4). The great electron scattering ability of the ThO₂ allowed it to be identified even within deposits of lead phosphate. The differences in appearance of the ThO₂ and lead phosphate were particularly apparent in unstained preparations (Fig. 4).

Corneas from 6- and 7-day-old rabbits, the age at which pinocytotic transport can first be demonstrated,14, 15 showed a slight reaction along the lateral cell margins, particularly in the basal half of the cells (Fig. 5). Vesicles and vacuoles which contained ThO₂ in these preparations again contained no ATPase activity.
Fig. 11. In the basal portion of the corneal epithelium, fixed for only 5 minutes in glutaraldehyde prior to incubation, lead phosphate end product is only in the intercellular spaces of the basal and intermediate layers. The basal membrane of the basal cells is negative, as is the basement lamina. (Original magnification ×10,300.)

In control preparations, no lead phosphate was seen after incubation without substrate. In preparations in which other substrates were substituted for ATP, slight reaction was seen in the lateral margins of endothelial cells. The reaction was identical with ADP, AMP, CTP, IDP, or β-glycerophosphate as substrate (Figs. 6 to 10). Under altered fixation conditions (i.e., when fixation was carried out for 5 minutes in 2 per cent glutaraldehyde or when glyoxal or hydroxyadipaldehyde was used rather than glutaraldehyde), the same localization of final product was seen with ATP as substrate.

After brief glutaraldehyde fixation, the adult corneal epithelium showed ATPase activity along the margins of all the cells except the apical surface of the outermost squamous layer and the basal surface of the basal layer (Fig. 11). Longer fixation apparently destroyed this activity in the adult cornea. Seven-day-old corneas, fixed for as long as 30 minutes, showed some ATPase activity in the epithelium (Fig. 12).

Discussion

The biochemical demonstration in many tissues of ATPases synergistically activated by Na⁺ + K⁺, together with studies showing that activity of these enzymes was cor-
related with rates of cation transport and was inhibited by ouabain. Stimulated numerous attempts to localize sites of these enzymes by histochemical methods. An interesting finding in some of the histochemical studies was that the so-called pinocytotic vesicles of certain capillaries, smooth muscle cells, some serosal cells, and Schwann cells demonstrated an intense nucleoside phosphatase activity. This activity, seen with a variety of nucleoside di- and triphosphate substrates, appeared to be localized on the inner surface of the vesicles. From these results, it was suggested that the phosphatase activity seen in such locations might be due to the activity of the so-called pump enzymes or might be related in some other way to the “transport” activity of the vesicles.

The first of these suggestions has always been somewhat controversial. As early as 1961, Novikoff reported that histochemical phosphatase activity was insensitive to ouabain, and, with few exceptions, most other workers have confirmed that histochemical activity is not activated by monovalent cations or inhibited by ouabain. Although activation by monovalent cations was not demonstrable under histochemical conditions of incubation, it was possible that the magnesium-dependent activity of this enzyme might still be demonstrated. Even this possibility appears unlikely in view of the relative sensitivity of the Mg-dependent activity to inhibition by -SH reagents and the fact that both aldehyde fixatives and lead ions are sulfhydryl inhibitors. The limited data available suggest that activity of the enzyme is strongly inhibited by lead and by brief exposures to dilute formaldehyde.

Such an argument against the identity of the enzyme demonstrated histochemically with the so-called pump enzymes is not necessarily conclusive, since only traces of chemically demonstrable activity need remain after fixation for activity to be demonstrated in situ, if the histochemical method used is sufficiently sensitive. Unfortunately, the sensitivity of the Wachstein-Meisel technique is not known. In the end, perhaps the strongest argument against identification of the histochemical activity with pump enzymes is that, chemically, regardless of activating ions, the pump enzyme is quite specific for ATP as substrate and is strongly inhibited by calcium. The histochemical activity, on the other hand,
is relatively nonspecific in its substrate requirements and, in at least one site, appears insensitive to calcium inhibition.\textsuperscript{32}

While pinocytosis has been shown to be an energy-dependent process,\textsuperscript{33, 34} the surface attachment and initial uptake phases may not require energy.\textsuperscript{34, 35} Certainly, energy-dependent electrolyte transport has never been shown to be directly associated with pinocytotic vesicles even though changes in electrolyte concentration at the cell surface can stimulate vesicle formation.\textsuperscript{33, 36} Moreover, the over-all importance of vesicular activity to cell transport processes remains problematic, for rapid, directed, net transport activity by pinocytotic vesicles has been clearly demonstrated in only one site, the corneal endothelium.\textsuperscript{2-7}

Although the vesicles of the myocardial capillary endothelium have been shown to take up colloidal markers and move them across the cell,\textsuperscript{37-39} the loading and transit times for these vesicles, although incompletely determined, appear to be too slow to account for significant net fluid transport.

The present study, combining the use of colloidal tracers, previously used to study transport in the corneal endothelium,\textsuperscript{7-11} and histochemical techniques to localize ATPase activity in this cell layer, clearly demonstrates that the pinocytotic vesicles engaged in transport exhibit no nucleoside phosphatase activity. However, the localization of activity in the lateral margins of the endothelial cells is identical with that found in the gall bladder,\textsuperscript{40} colon,\textsuperscript{41} kidney,\textsuperscript{42} toad urinary bladder,\textsuperscript{43} ciliary epithelium,\textsuperscript{43} and other sites in which electrolyte transport occurs.\textsuperscript{29} Although it seems highly unlikely that the histochemical activity seen is due to surviving cation-activated ATPase, it is interesting that in all sites examined in which active electrolyte transport (and, therefore, concomitant fluid transport) occurs there is a localization of ATPase activity on the lateral cell margins. Unless these findings are taken to be coincidental, it may be inferred that the enzyme so demonstrated has some connection, as yet undefined, with transport processes.

Since the present study demonstrates that pinocytotic vesicles engaged in transport show no ATPase activity, it is difficult to explain the results obtained by Barnett and co-workers\textsuperscript{14, 15} and by Carasso and co-workers.\textsuperscript{3} There certainly is, with the possible exception of the myocardial capillaries discussed above,\textsuperscript{35-38} a distinct lack of data indicating that the vesicles studied by Barnett and co-workers\textsuperscript{14, 15} and Carasso and co-workers\textsuperscript{3} have any connection with transport. It is most likely that vesicles which exhibit such relatively nonspecific nucleoside phosphatase activity have a digestive or permanently absorptive function, as in the invaginations of the cell surface for protein absorption described by Roth and Porter.\textsuperscript{44} There is little doubt that the vesicles in the ciliate \textit{Campanella umbellaria} studied by Carasso and co-workers\textsuperscript{3} are pinocytosing material from the food vacuole for subsequent digestion. Indeed, Rostgaard and Barnett themselves suggest the possibility of such a digestive or absorptive function for the fixation-resistant phosphatase of intestinal smooth muscle.\textsuperscript{4}

It appears that the corneal endothelium, because of its peculiar avascular condition, has adapted the general cellular absorptive process of pinocytosis to a specific transport function. From the data presented here and from the studies of Kaye and Donn\textsuperscript{16} on ouabain effects on pinocytosis, it seems that the pinocytotic transport activity in the corneal endothelium may be dependent upon a functioning electrolyte transport system in the lateral margin of the cells.

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REFERENCES


