BBA 75161

LEVELS OF $(Na^+ + K^+)$-ACTIVATED AND Mg^{2+}-ACTIVATED ATPase ACTIVITY IN BOVINE AND FELINE CORNEAL ENDOTHELIUM AND EPITHELIUM

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(Received February 19th, 1968)

SUMMARY

1. An active transport mechanism appears to be at least in large part responsible for the maintenance of corneal deturgescence. The site of the mechanism has been in dispute, with the endothelial or epithelial limiting layers favored by different sorts of evidence.

2. Fresh bovine and feline corneal endothelium and epithelium were assayed for $(Na^+ + K^+)$-activated ATPase and for Mg^{2+}-activated ATPase (EC 3.6.1.4) activities by quantitative biochemical methods, employing ouabain at a concentration of 0.1 mM. Epithelium was collected by three different methods. A ouabain inhibition curve was also determined for the enzyme of bovine endothelium.

3. Bovine endothelium was compared with epithelium collected by the most satisfactory method. In terms of enzymatic levels based on tissue nitrogen, the endothelium had 6.4 times as much $(Na^+ + K^+)$-activated activity, and 5 times as much Mg^{2+}-activated activity, as the epithelium.

4. The fewer determinations for the feline cornea indicate that the $(Na^+ + K^+)$-activated activity levels for both the endothelium and epithelium are within the same ranges as those of the bovine cornea.

5. The ouabain inhibition curve for the bovine endothelial enzyme matches rather closely the curve from other work for the rate of swelling of the rabbit cornea when different concentrations of ouabain were perfused on the endothelial side of the cornea in culture.

6. The present results fit well with the results of recent metabolic studies of others on corneal endothelium and epithelium, which support the endothelium as the major site of the mechanism that maintains corneal deturgescence.

INTRODUCTION

Although it is fairly generally agreed that an active secretory process is largely responsible for maintaining the relative dehydration of the cornea[1], the site of the mechanism has been in dispute. On the basis of various types of observations, the endothelium (mesothelium, or mesenchymal epithelium) has been said to be the

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primary site of the mechanism that removes water[2–5]. Electron-microscopic evidence has been used to implicate the endothelium in the process of transport by means of pinocytotic vesicles[6–12], as well as to deny that it could act in transport, owing to the absence of vacuoles and infolded cell membranes[13]. The epithelium has also been held to be the primary site, because of the existence of transcorneal and transepithelial potentials, and the movement of Na^+ across the cornea from the epithelial to the endothelial side[14–18]. The stroma has been shown to be capable of maintaining its own state of dehydration when portions of it are partially isolated experimentally from the endothelium and epithelium[19].

$(Na^+ + K^+)$-activated ATPase has been linked to active cation transport in cell membranes of many kinds of cells, with accompanying water secretion in many epithelial cell types[20]. BONTING, SIMON AND HAWKINS[21] reported ATPase levels of corneal epithelium, stroma and endothelium of the cat. $(Na^+ + K^+)$-activated ATPase activity comprised 5.1 % of the total ATPase activity in the epithelium and 33 % of the total activity in the endothelium. Nevertheless, on the basis of tissue wet weight, the level of $(Na^+ + K^+)$-activated ATPase activity of the epithelium was approximately twice that of the endothelium. Stromal levels were listed as being below a minimal measurement level. LANGHAM[22] found that the collagen of the rabbit corneal stroma, comprising 0.9 of the corneal mass, has low metabolic activity compared with the epithelium and endothelium. LANGHAM AND KOSTELNIK[23] studied the ATPase of bovine and rabbit epithelium of corneas in some cases stored at 4° for up to 72 h. The ouabain-sensitive enzymatic activity amounted to about 10 % of the total when the high concentration of 1.0 mM ouabain was employed. No determinations were made for the endothelium. The present work reports the comparative quantitative levels of Mg^{2+}-activated and Mg^{2+}-dependent, $(Na^+ + K^+)$-activated ATPase activities for bovine and feline corneal endothelium and epithelium, and a ouabain inhibition curve for the ATPase of bovine corneal endothelium.

MATERIALS AND METHODS

Materials and preparation

Cattle eyes were obtained from a commercial slaughter house. Eyes were placed on cracked ice in an insulated container with the corneas upward and in contact only with moist air, in the minimum time feasible after the death of the animals. Scraping of endothelium and epithelium began 20 to 25 min after the eyes were placed on ice. Beaver blades (No. 64) with rounded cutting edge were used under a dissecting microscope, and the tissue was collected in a measured amount of glass-redistilled water at 0°. Any pieces of stroma that appeared in the water were picked out and discarded. About 20 eyes were used in each assay. In the course of 5 experiments, 10 determinations on epithelium and 9 on endothelium were made. Because of its delicacy, endothelium was collected first from excised corneas in experiments in which both sides were to be scraped. The tissue was homogenized by hand in ice-cold glass homogenizers. Material in the various experiments was placed in the homogenizers from 1.5 to 5 h after the death of the animals.

Endothelium and epithelium from the eyes of 18 cats were prepared as described for the bovine eyes. About 10 h elapsed while the tissue was added. Each type of tissue was pooled for enzymatic determination.

Serial sections of scraped corneas cut at 10 μ, employed to see if the epithelium was completely removed, showed that much of the epithelium was resistant to this sort of scraping. Additional determinations were therefore made on corneal epithelium collected by other methods. The endothelium was first removed, and both corneal surfaces were carefully washed with ice-cold water. A motor-driven, wire contra-angle derm-abrasion brush routinely employed in skin surgery was used to remove epithelium in water at 0°. 5 determinations were made on bovine epithelium.

During the course of this work it became apparent that if the endothelium were removed and the cornea were placed with that surface on an iced Syracuse dish for several minutes, the epithelium loosened from the stroma so that it could easily be scraped off with the Beaver blade. 10 Enzymatic determinations in 2 experiments on bovine epithelium and 9 determinations in 3 experiments on feline epithelium, collected in this manner, were made. All material from the bovine corneas was placed in homogenizers less than 2.5 h after death of the animals, and from feline corneas, in less than 1.25 h.

ATPase assays

The assay medium of BONTING, SIMON AND HAWKINS[21], modified by two changes, was used throughout. $MgSO_4 \cdot 7H_2O$ was substituted for $MgCl_2 \cdot 6H_2O$, and the amount of Mg^{2+} was doubled to 2 mM, to make it equal to the ATP concentration. Ouabain was employed at 0.1 mM concentration, as in the BONTING medium. In 7 assays in which Na^+ and K^+ were omitted from the media, results were virtually the same as the results with ouabain, except for one case, in which the activity level with ouabain was a little lower. The results reported are based on ouabain inhibition. Constriction micropipettes were used to transfer 100 μl of homogenate and 200 μl of assay medium to each tube, kept in cracked ice. Assay tubes were capped and incubated for 1 h in a water bath at 38°, with shaking by hand every 15 min. At the end of the incubation period they were returned to ice, and 1.5 ml of cold 10% (w/v) trichloroacetic acid was added to each tube. Tissue blank tubes remained in ice and received the trichloroacetic acid at the start of the incubation period. All tubes were centrifuged for 10 min at 2700 rev./min in a refrigerated centrifuge, 1.2 ml of supernatant was transferred from each tube to a Coleman cuvette tube, 1.2 ml of ferrous sulfate–ammonium molybdate color reagent was added, and the color was allowed to develop for 0.5 h. Readings were made at 700 mμ on a Coleman spectrophotometer. The results from 4 similarly prepared tubes were averaged for each assay or tissue blank. Phosphate standard dilutions were prepared and read with each experiment, and the experimental phosphate mM concentrations were read from the resulting curves. These were converted to actual μmoles per mg of tissue nitrogen by calculation.

A ouabain inhibition curve was determined for the ATPase of bovine endothelium, prepared as described above. Ouabain was employed at stepwise concentrations from 1.0 mM to 0.01 μM.

Nitrogen determinations

Nitrogen content was determined for each homogenate, using a modified CONWAY diffusion micro-method adapted from PARKER[24], with some changes based on our micro-KJELDAHL method[25]. Nine 100-μl aliquots of each homogenate were digested and 6 to 9 titrated.

RESULTS

Table I contains the levels of ATPase activity determined for bovine corneas. Expt. A consists of the determinations made on tissue scraped from the eyes immediately after removal from the cold box, with no other treatment. Based on tissue nitrogen, the endothelium had 12.8 times as much $(Na^+ + K^+)$-activated activity and

TABLE I

BOVINE ENDOTHELIAL AND EPITHELIAL ATPase

Data are presented as μmoles of phosphate liberated per mg of tissue nitrogen per h of incubation, followed by the standard errors. Percentages represent the portion of total ATPase activity that was ouabain-sensitive ($(Na^+ + K^+)$-activated). Expt. A, tissue scraped from corneas with Beaver blade; means of 9 determinations for endothelium and 10 determinations for epithelium. Expt. B, epithelium removed from corneas with motor-driven wire brush; means of 5 determinations. Expt. C, endothelium removed, corneas chilled on ice-cold glass for several minutes, and epithelium scraped off with Beaver blade; means of 10 determinations.

Expt.	*Mg^{2+}- plus $(Na^+ + K^+)$-activated ATPase*		*Mg^{2+}-activated ATPase*	
	Endothelium	*Epithelium*	*Endothelium*	*Epithelium*
A	13.5 ± 1.9 (33%)	1.1 ± 0.32 (12%)	27.5 ± 1.5	7.5 ± 0.8
B		1.0 ± 0.11 (26%)		2.8 ± 0.08
C		2.1 ± 0.27 (28%)		5.5 ± 1.1

3.7 times as much Mg^{2+}-activated activity, as the epithelium. Expt. B contains the values obtained for epithelium taken from corneas from which the endothelium was scraped and discarded, and the corneas gently washed on both sides, before the epithelium was removed with the motor-driven wire brush. Serial sections of one of these corneas showed that the epithelium was completely removed. Expt. C contains the values obtained for epithelium from corneas from which the endothelium was

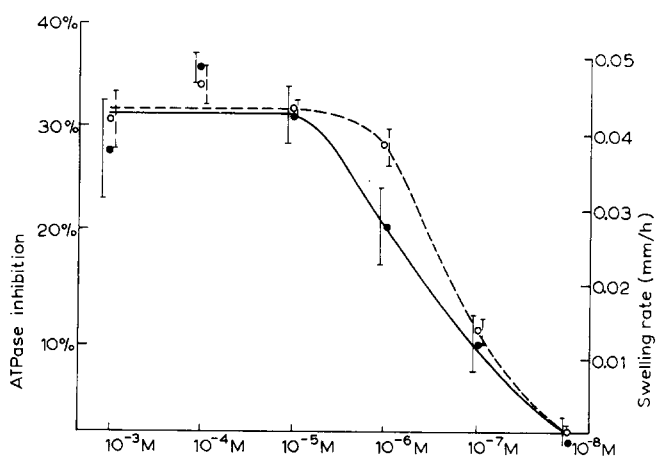

Fig. 1. Solid circles and line: Inhibition curve of bovine corneal endothelial total ATPase at various molar concentrations of ouabain. Open circles and dashed line: rate of swelling of the rabbit cornea in culture when the corresponding concentrations of ouabain were perfused on the endothelial side (data kindly supplied by Dr. S. MISHIMA). Vertical bars: standard errors, based on 4 duplicate assay tubes for ATPase and 5 corneas for swelling rate.

scraped and discarded and the corneas washed and placed on glass at 0° for several minutes before the epithelium was scraped off. Sections of both a bovine and a feline cornea treated in this manner showed that the epithelium was cleanly and completely removed. The previously determined levels for endothelium are 6.4 times higher for $(Na^+ + K^+)$-activated activity and 5.0 times higher for Mg^{2+}-activated activity than these epithelial levels.

Fig. 1 shows the ouabain inhibition curve for ATPase of the bovine corneal endothelium, plotted against data kindly supplied by Dr. S. MISHIMA for the rate of swelling of the rabbit cornea when the corresponding concentrations of ouabain were perfused on the endothelial side of the cornea in culture.

Table II contains the values obtained for feline endothelium and epithelium. Expt. A consists of the determinations for tissue scraped from eyes immediately after enucleation and kept in water at 0° until the material from 18 cats could be pooled. Expt. B contains the results of determinations on epithelium removed after the corneas were chilled and in which the preparation time was very short.

TABLE II

FELINE ENDOTHELIAL AND EPITHELIAL ATPase

Data are presented as μmoles of phosphate liberated per mg of tissue nitrogen per h of incubation, followed in Expt. B by the standard errors. Percentages represent the portion of total ATPase activity that was ouabain sensitive. Expt. A, tissue scraped from corneas with Beaver blade, pooled tissue from 18 cats. Expt. B, endothelium removed, corneas chilled, and epithelium scraped. Means of 9 determinations.

Expt.	*Mg^{2+}- plus $(Na^+ + K^+)$-activated ATPase*		*Mg^{2+}-activated ATPase*	
	Endothelium	*Epithelium*	*Endothelium*	*Epithelium*
A	7.9	0.0	6.7	6.4
B		2.2 ± 0.6 (6.6%)		31.2 ± 3.2

DISCUSSION

The relative $(Na^+ + K^+)$-activated ATPase activities of endothelium and epithelium reported here differ from those of BONTING, SIMON AND HAWKINS[21] for the cat cornea. Because of the delicacy of the endothelium and attendant difficulties in freeing it from aqueous humor, we do not believe that it is possible to obtain a meaningful wet weight for this tissue.

The enzymatic levels for the epithelium in Table I, Expt. A, may be influenced by the inclusion of some blood cells in assays, since these corneas were not washed before scraping. Not all of the epithelium was removed from these corneas in the scraping process and the preparation time was relatively long, undoubtedly resulting in some loss of enzymatic activity. However, in this series the preparation time was the same for endothelium and epithelium. The levels in Expt. B are low, but it is not apparent what factors in the motor-driven wire-brush treatment are responsible. The epithelium in Expt. C had the shortest preparation time of any of the bovine material. Nevertheless, the epithelial level of $(Na^+ + K^+)$-activated activity was very much lower than the endothelial level in Expt. A, determined in experiments in which

the average preparation time was longer. The feline material in Table II, Expt. A, was collected over a 10-h period, during which enzymatic loss undoubtedly occurred, in greater amount in the material collected earlier in this period. Nevertheless, the value determined for endothelium was the same as the two lowest individual values determined for bovine endothelium. The epithelial value for $(Na^+ + K^+)$-activated ATPase activity in Expt. B is nearly the same as that determined for bovine epithelium with the same method of preparation. The preparation time for this tissue was the shortest of any of the experiments. Only with regard to the Mg^{2+}-activated ATPase activity does there appear to be a species difference.

GREEN[16–18] has reiterated the viewpoint that an active transport pump exists in the corneal epithelium but not in the endothelium. This belief rests on the demonstration of Na^+ movement from the epithelial side to the endothelial side of the rabbit cornea in culture chambers[14,16,17] and on the demonstration of an epithelial potential, negative on the tear side[16–18,26–30]. Using a microelectrode method, KIKKAWA[31–33] obtained different results for corneal potentials. Epithelial potentials were found to be negative on the stromal side. A small endothelial potential was measured, and the stromal side was always negative with reference to the aqueous humor. Other workers have not been able to demonstrate a potential across the endothelium with their methods. In any case, several of the reports on the epithelial potential[26,28] strongly emphasize that there is no relation between the potential difference across the corneal epithelium and the state of hydration of the corneal stroma.

Metabolic and biochemical studies have contributed several lines of evidence in support of the endothelium as the major site of the activity that maintains the normal state of hydration of the cornea. The data of HARRIS AND NORDQUIST[34] fit the concept of an active endothelial transport system that moves both Na^+ and water into the aqueous humor. During temperature reversal recovery, the actual amount (not the concentration) of Na^+ in rabbit corneas decreased, while Na^+ concentration in the aqueous humor rose.

ITOI, KOMATSU AND TANDA[5] favored a water-pump mechanism because they found that the process was not susceptible to ouabain inhibition. There is abundant evidence, however, that ouabain does affect this process[1,11,23,35–37]. The experiments of MISHIMA AND KUDO[38] and TRENBERTH AND MISHIMA[37], utilizing rabbit corneas in a perfusion culture chamber, favor an endothelial site for the pump mechanism that governs stromal dehydration. With proper medium, normal corneal thickness could be maintained, and temperature reversal recovery could be demonstrated. When carefully removed, the epithelium was shown to play no role in these phenomena, but the endothelium was shown to be essential. Furthermore, inhibition of the endothelium by ouabain, removal of the energy source from the endothelial side (reversible by resupply), or removal of Ca^{2+} from the endothelial side, all caused corneal hydration and swelling. In the last case, electron microscopy showed that endothelial cell boundaries had broken down[39]. With similar culture methods, TAKAHASHI AND MISHIMA[40] have shown that anoxia of the endothelial side, but not of the epithelial side, resulted in swelling of the cornea. With both sides anoxic and the cornea swollen, some recovery was noted when the endothelial surface was resupplied with 7 % O_2, 5 % CO_2 and balanced N_2.

The present enzyme studies do not in themselves tell which corneal limiting layer is more important in maintaining stromal deturgescence, but they fit well with

the results just cited. The similarity of the ouabain inhibition curve for the enzyme and the curve for ouabain effect on corneal hydration and thickening (Fig. 1) make it seem likely that the enzyme is associated with the mechanism of relative dehydration.

ACKNOWLEDGEMENTS

I wish to thank Miss ELISABETH MAAG, Mrs. IRMGARD WOOD, and Mr. MASAO OKUMOTO for their valuable technical assistance. I wish to thank Dr. WILLIAM MCEWEN for many helpful discussions. This work was supported in part by Research Grant No. NB-06207 from the National Institutes of Health, U.S. Public Health Service.

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