Biochimica et Biophysica Acta, 307 (1973) 557–562

BBA 76317

FLUID TRANSPORT, ATP LEVEL AND ATPase ACTIVITIES IN ISOLATED RABBIT CORNEAL ENDOTHELIUM

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(Received January 8th, 1973)

SUMMARY

The effect of certain biochemical parameters on transendothelial fluid transport has been studied. Cellular ATP level and $(Na^+ + K^+)$-activated as well as Mg^{2+}-activated ATPase activities were measured by ultramicrotechniques using individual rabbit corneal endothelium after they had been subjected to *in vitro* perfusion with solutions fully supplemented or deficient singly or severally in glucose, adenosine and glutathione (GSH). With the complete medium, the transport system operates *in vitro* for approx. 6 h. Deletion of glucose alone, glucose and adenosine or glucose, adenosine and GSH brings about a cessation of fluid transport after 3.5 h, 2 to 2.5 h and 0.5 to 1 h, respectively. A marked decrease (62%) of the endothelial ATP level, however, occurs only when all metabolites are omitted. The favorable effect of GSH on transport activity is attributable to its capacity to sustain cellular ATP rather than to protect the functionality of $(Na^+ + K^+)$-activated ATPase. Adenosine, in the presence of GSH, maintains normal ATP levels and, additionally, exerts a protective effect on Mg^{2+}-activated ATPase and possibly also on $(Na^+ + K^+)$-activated ATPase.

INTRODUCTION

The energy requirement for the transport of materials across biological membranes is generally considered to be met by cellular high-energy phosphate metabolites[1,2]. An enzyme, located within membranes and designated by its cation requirements as an $(Na^+ + K^+)$-activated ATPase, appears to be intimately linked to the transport process[3]. The involvement of these factors in the cornea has been observed by Rogers[4], who noted the similarity between the curves for ouabain-inhibited endothelial $(Na^+ + K^+)$-activated ATPase activity of bovine cornea and the rate of swelling of rabbit corneas perfused with the same glycoside[5]. Other relevant factors have been investigated by Dikstein and Maurice[6], who have demonstrated that cold-swollen corneas will effectively deturgesce *in vitro* in the combined presence of exogenous GSH and adenosine.

The present study examines the relation between *in vitro* fluid transport across rabbit corneal endothelium and both cellular ATP level and $(Na^+ + K^+)$-activated as well as Mg^{2+}-activated ATPase activities. Measurement of these biochemical parameters on a pair of rabbit corneal endothelia from the same animal was achieved

by adapting existing analytical methods and modifying others to the ultramicro level. The results show that the influence of the above metabolites on fluid transport can be attributed to a positive effect on one or more of the biochemical parameters studied.

METHODS

Fluid transport was measured according to techniques developed earlier[6–8] and adapted[9] to the present purposes. The animals used were albino female rabbits 4–6 months old and weighing 2.5–3 kg. Two endothelia supported by stroma were obtained by scraping free of epithelium a pair of corneas taken from the same rabbit. After mounting the preparations in separate perfusion chambers at 37 °C, the endothelial surfaces were perfused at a rate of 49 μl/min with a complete medium modified from that of Dikstein and Maurice[6] and consisting of salts and nutrients at the following concentrations in mM: NaCl, 110; $NaHCO_3$, 39; $KHCO_3$, 3.8;KH_2PO_4, 1.0; $MgSO_4$, 0.78; $CaCl_2$, 1.7; glucose, 6.9; adenosine, 5.0; and GSH, 0.24. The endothelial side of the preparations carried a pressure head of 20 cm H_2O. The denuded epithelial sides were bathed concurrently with a stationary basal salt solution containing salts identical to those in the complete medium and sucrose equivalent on an osmolal basis to the combined nutrients in the complete medium. Stromal thickness, which was monitored with a specular reflexion microscope[6,10], increased under these conditions as a result of fluid imbibition across the epithelial side. After a suitable interval, fluid transfer across this surface was suppressed by replacing the basal solution with silicone oil. Under these conditions, any ensuing change in stromal thickness is attributable[5,11] to fluid movement across the endothelium and average rates of transendothelial fluid transport can be estimated from the slope of plots of stromal thickness *vs* time (*cf.* Fig. 1). When desired, perfusion without previous swelling was achieved by placing silicone oil on the denuded epithelial side of the stroma. Continued perfusion of one preparation with complete medium constituted a control for the test preparation which had the complete medium replaced by a test solution.

At the termination of this part of the experiment, the single layer of endothelial cells from each preparation was collected separately by scraping it into 50 μl cold distilled water, rapidly freezing, thawing and homogenizing in a 1-ml conical centrifuge tube fitted with a stainless steel pestle for three 5-s intervals with two 3-s interruptions for rechilling in an ice–water bath. A 10-μl portion of the homogenate was taken rapidly into an equal volume of cold 1.0 M $HClO_4$, mixed and frozen. It provided six 3-μl samples which were used within 2 h for the determination of ATP[12]. Results agreed within $\pm 5\%$, with a recovery of 96.5%. Total Mg^{2+}-activated *plus* $(Na^+ + K^+)$-activated ATPase activity, 10^{-4} M ouabain-inhibited ATPase activity and endogenous P_i were determined with 5-μl portions of homogenate which were stored until use at -84 °C for 20 to 40 h. The basic method of Bonting *et al.*[13] was employed but with levels of ATP and Mg^{2+} elevated to 6 mM in a final volume of 25 μl. From the absolute values of P_i, hydrolysis of ATP during 60 min of incubation at 37 °C never exceeded 60% of that theoretically available. Rate curves for both Mg^{2+}-activated and $(Na^+ + K^+)$-activated ATPase were determined up to 90 min and revealed that the rate of P_i release becomes essentially constant after 40–45 min. This confirms the observation of Nørby[14] who has shown

that steady-state kinetics can be attributed to the reformation of ATP *in situ* by the action of adenylate kinase on ADP. With the small amounts of protein present deproteinization could be omitted[15] without affecting the results. After correction for P_i, the difference between total activity and ouabain-inhibited activity was taken as a measure of $(Na^+ + K^+)$-activated ATPase activity. Duplicates agreed within $\pm 6\%$. Since endothelia of young rabbits have been shown histochemically[16] to contain very few mitoses, cellular DNA content was considered constant. It was determined[17] for each endothelium in quadruplicate with an agreement of $\pm 8\%$ and used to normalize ATP and ATPase values.

RESULTS

Preliminary experiments revealed that the biochemical data could differ by a factor of 2 between endothelia from different animals, but that the data from a pair of endothelia from the same animal agreed approximately within the error of determination of each parameter. To illustrate this, the ranges of values for controls of 19 experiments were as follows: ATP, 360 to 700 pmoles/μg DNA; Mg^{2+}-activated ATPase activity, 203 to 390 nmoles P_i/h per μg DNA; $(Na^+ + K^+)$-activated ATPase activity, 48 to 93 nmoles P_i/h per μg DNA. In four pairs of controls, however, where values from one of each pair were randomly taken as 100%, the biochemical data showed the following agreement (mean $\pm$S.E.): ATP, $102 \pm 3\%$; Mg^{2+}-activated ATPase activity, $102 \pm 3\%$; $(Na^+ + K^+)$-activated ATPase activity, $98 \pm 8\%$. The relatively large deviations for the $(Na^+ + K^+)$-activated ATPase are not surprising since this ouabain-inhibited fraction represented only approx. 19% of the total ATPase activity. From these data, it is apparent that a given pair of endothelia from the same rabbit can be used to examine the effect of environmental changes on these biochemical parameters. In view of the ranges for these values, data for the test preparations are expressed as percent of the paired control values (*cf.* Fig. 1).

Stromal thickness is assumed[11,18] to reflect the combined effects of two opposing processes: passive imbibition, *i.e.* fluid leak into the stroma, which increases thickness, and endothelial transport, which decreases thickness. Perfusion of the test preparations was terminated closely following that time (*cf.* Fig. 1) when the rate of imbibition began unquestionably to exceed that of transport. Under conditions of active deturgescence the initial rate of fluid transport was about 6 μl/h per cm^2, while the imbibition rate, determined from the last few thickness readings of the test preparations, was 4.0 to 4.5 μl/h per cm^2. Both values are essentially in agreement with those reported in the literature[11,18].

Fig. 1 shows the effect of omitting from the perfusion medium either glucose alone or together with either adenosine or adenosine and GSH. For a swollen preparation to effect deturgescence in the absence of all nutrients in the perfusing solution, energy must be supplied by tissue reserves. As shown in Fig. 1, Expt I, endogenous reserves sustained transport during perfusion with basal solution for less than 1 h. Cellular ATP was depleted 62% and activities of Mg^{2+}-activated ATPase and $(Na^+ + K^+)$-activated ATPase were reduced 16% and 33%, respectively. That the swelling procedure employed did not *per se* affect the ATP level was established in three separate experiments in which test preparations were swollen for 30, 45 and 60 min and then compared to their respective unswollen controls; the mean test

ATP value was 94.3±5.9% of the control. Similarly, perfusion of an unswollen preparation with complete medium for up to 3.5 h did not appreciably affect the biochemical parameters. This was established in two experiments where the final values in percent compared to those of the control member of the pair (taken for

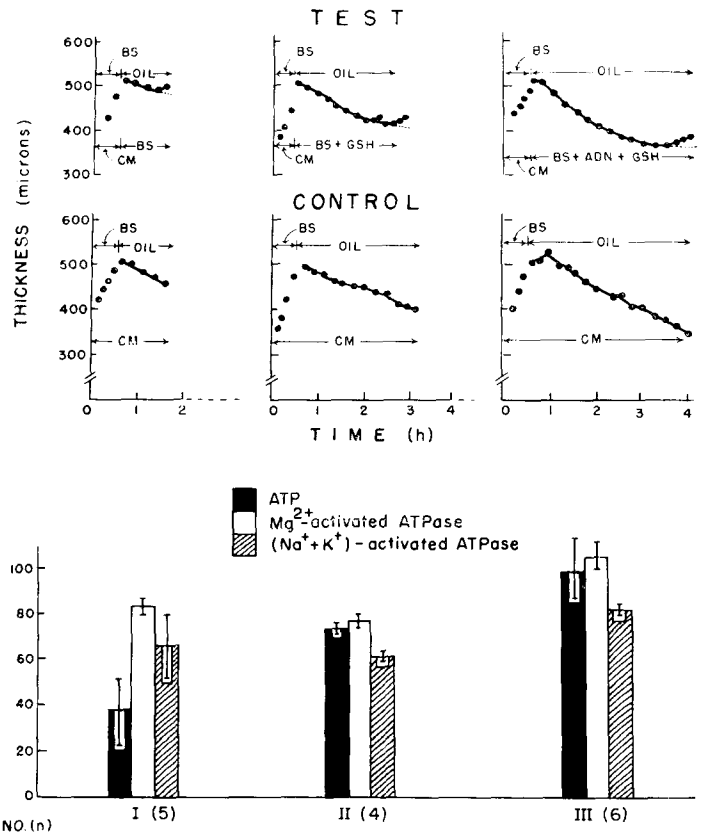

Fig. 1. Effect of various metabolite omissions on the rate of endothelial fluid transport, ATP level and ATPase activities. Each cornea from a given pair of rabbit eyes was freed of epithelium, mounted in a chamber and perfused according to techniques described in the text. Legends above and below the experimental curves of thickness *vs* time denote the fluids in contact with the outer (epithelial) and the inner (endothelial) surfaces, respectively, and the intervals during which they were employed. Abbreviations are as follows: ADN, adenosine; BS, basal solution; CM, complete medium. Individual thickness readings (●) were accurate to ± 1 μm. At the time of termination of the experiment, the rate of change in thickness per unit time, equivalent to the rate of transendothelial fluid movement per unit area, differed between tests and controls by more than 45 μm/h or 4.5 μl/h per cm^2. Dotted lines extrapolating the terminal parts of the curves emphasize this fact. The thickness *vs* time curves are from one typical experiment of each group, while the biochemical data represent all experiments within each group. Numbers in parentheses following Expt No. indicate the number (n) of similar experiments performed. Bar data represent the mean test value ±S.E. The following pooled data from the 15 controls are provided for general orientation: ATP, 571±34 pmoles/μg DNA; Mg^{2+}-activated ATPase, 257±15 nmoles P_i/h per μg DNA; ($Na^+ + K^+$)-activated ATPase, 53±5 nmoles P_i/h per μg DNA; DNA, 1.65±0.09 μg/endothelium. The use of DNA to normalize all values was based on the assumed constancy of this component in young rabbits with essentially non-mitosing endothelial cells[16].

analysis immediately after *in vitro* mounting) were: 104 and 100% ATP; 103 and 93% Mg^{2+}-activated ATPase activity; 92 and 79% $(Na^+ + K^+)$-activated ATPase activity.

The effect of depriving the tissue of all exogenous nutrients except GSH is shown in Expt. II. Endothelial transport was maintained longer than with basal solution alone but ceased after 2.5 h when the ATP level was still 74% of the control level. Mg^{2+}-activated and $(Na^+ + K^+)$-activated ATPase activities were reduced 23% and 39%, respectively.

The plot of thickness *vs* time in Expt III shows that although perfusion with adenosine and GSH results in an initial rate of deturgescence nearly comparable to that of the control, the deturgesced state is not sustained in the absence of glucose. At the point of termination, after approx. 3.5 h of perfusion, endothelial fluid transport was being outpaced by fluid imbibition. The bar data show, however, that the ATP level of the endothelium was essentially normal. Enzyme activities also indicate no functional impairment of the Mg^{2+}-activated ATPase and a questionable reduction of only 18% in $Na(^+ + K^+)$-activated ATPase activity.

DISCUSSION

Deturgescence of swollen endothelial preparations deprived of exogenous metabolites is severely limited and, as would be expected of an energy-requiring process, markedly depletes the cellular ATP. This loss of ATP, however, can be reduced nearly 50% by GSH supplementation. This fact most probably underlies the prolongation of pump function observed here and previously by others[6] when this single metabolite is available. Since GSH appears to have had no significant effect on protecting the functionality of either Mg^{2+}-activated or $(Na^+ + K^+)$-activated ATPase in these experiments, one may reasonably conclude that the beneficial effect exerted by GSH on fluid transport involves some mechanism that generates ATP.

Adenosine, in the presence of GSH, can maintain cellular ATP and Mg^{2+}-activated ATPase activity at essentially normal values for as long as the transport mechanism operates. The fact that transport will cease prematurely in spite of the normalcy of these parameters suggests that some other parameter(s) not investigated is pertinent to net outward fluid movement. It seems unlikely that an 18% reduction in activity of $(Na^+ + K^+)$-activated ATPase could be the major cause of pump failure. This observed decrease in activity, aside from its questionable significance, can be calculated from published data[4] to be comparable to that which would result from the presence of $7 \cdot 10^{-8}$ M ouabain. This concentration would theoretically cause a fluid leakage into the stroma at the rate of 0.8 μl/h per cm^2 (ref. 5) rather than the experimentally observed rate of approx. 4.5 μl/h per cm^2.

In spite of these protective effects of both GSH and adenosine, pump function is still not sustained beyond a few hours. Glucose, which was present in the medium of the control preparations, or a metabolic product(s) formed from glucose *in situ* appear(s) also to be necessary for additional prolongation of fluid transport. Yet, even perfusion with the complete medium will not maintain *in vitro* the pump function[6] or the electrical potential difference[19] for more than 5 to 6 h.

The possibility that the observed failure of fluid transport was due to cellular

damage and subsequent increased endothelial permeability caused by the conditions of the experiments has been considered. Experiments were therefore performed to measure the rate of ^{22}Na outflux from the stroma across the endothelium during perfusion with: (a) GSH; (b) adenosine and glucose; or (c) complete medium. No obvious difference in rates of ^{22}Na outflux were observed among the three conditions. Excluding small variations that may have existed but were beyond experimental detection, these observations support the premise that significant changes in passive permeability probably had not occurred but that the transport mechanism itself had ceased to function.

The present findings do not contradict the view that an adequate level of cellular ATP and a functional $(Na^+ + K^+)$-activated ATPase are requisites for transport activity. The premature cessation of pump activity that was observed when both of these parameters are within or near established norms probably reflects the need for other as yet undefined factors. The results also offer some insight into the beneficial effects that GSH and adenosine exert on the physiological behavior of corneal endothelium.

ACKNOWLEDGEMENTS

This work was supported by N.I.H. research grant numbers EY-00699 and EY-00727 from the National Eye Institute, and in part by Fight for Sight, Inc., N.Y.C. The authors acknowledge the competent technical assistance of Miss Pegri Varjabedian and Mr Stephen Preas, and the construction of perfusion chambers by Mr Heinz Rosskothen.

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