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LOCAL OSMOTIC COUPLING TO THE ACTIVE TRANS-ENDOTHELIAL BICARBONATE FLUX IN THE RABBIT CORNEA

KENNETH R. MAYES and STUART HODSON

Unit of Electron Microscopy, Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XW (U.K.)

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Summary

The present experiments investigate HCO_3^-, Cl^- and fluid fluxes across partially destromalised corneas. Although there is no net flux of Cl^-, there is a net flux of HCO_3^- across the endothelium from stromal side to aqueous side which is accompanied by a flux of water in the same direction. Bulk phase osmosis cannot account for the initiation of the flux of fluid. Local osmotic coupling between ions and water is postulated to occur in the preparation. The exudate is hypertonic to the bathing Ringer solution.

Introduction

The hydration of the cornea results from an equilibrium between the passive tendency of the stroma to swell [1,2] and an active process which dehydrates the stroma. This active process is located in the endothelium [3,4] and it has been demonstrated that the endothelium moves fluid across itself from the stromal side to the aqueous humour [5]. The mechanism responsible for this fluid translocation which maintains corneal hydration appears to be a bicarbonate ion pump located in the endothelium [6,7]. The nature of the coupling between the bicarbonate ion flux and water flux is unclear. A role in stromal dehydration has also been asigned [8,9] to a Cl^- pump in the corneal epithelium. The in vitro contribution of the epithelium to the dehydration process is some 3% of that of the endothelium [8].

The present paper demonstrates that the flux of fluid out of the stroma across the endothelium is associated with a movement of HCO_3^- in the same direction and that there is local osmotic coupling between the ion and water fluxes.

Materials and Methods

Corneas (350—400 μm physiological thickness) taken from 3-month-old Dutch rabbits were partially destromalised (about one-half stromal thickness

removed from the anterior epithelial surface) to reduce subsequent obscuring of the endothelium. They were tied on to perspex rings, mounted in a 35°C water jacket and examined with a specular microscope after the method of Dikstein and Maurice [4]. The endothelial surface was perfused with a bicarbonate-glutathione Ringer solution [6] at a rate of 1.8 ml/h and a hydrostatic pressure of 12 cm water (about one-half physiological) was applied to that surface.

A known volume (400 μl) of the Ringer solution was pipetted on to the stromal surface and covered with silicone oil. The specular microscope could be focused down through the system so that the oil-Ringer interface, the Ringer-stroma interface and the endothelium could be seen clearly (Fig. 1) [5]. Some time (1—3 h) was required for the stromal thickness to stabilise because of initial imbalances of the stromal hydration. After this period, when the stromal thickness was seen to have equilibrated, the thickness of the Ringer layer overlying the anterior stromal surface decreased at a constant rate. Active translocation of fluid across the cornea from the overlying Ringer layer into the perfusing solution is occurring (phenomenon established by Maurice [5] and confirmed in this laboratory using the inert vinyl prosthesis in place of the cornea). The Ringer layer plus the silicone oil was now aspirated from the stromal surface using a fine catheter attached to a syringe. Recovery with this technique is greater than 98.7% [10].

A fresh, pre-warmed, known volume of Ringer solution (400 μl) was immediately pipetted on to the stromal surface and covered with silicone oil. Changes in the thickness of this Ringer layer were monitored with the specular microscope.

In three preparations, 10-μl samples of the Ringer in this layer were taken at intervals throughout the experiment so that its total CO_2 and Cl^- concentrations could be monitored. In four other experiments, 10-μl samples were taken

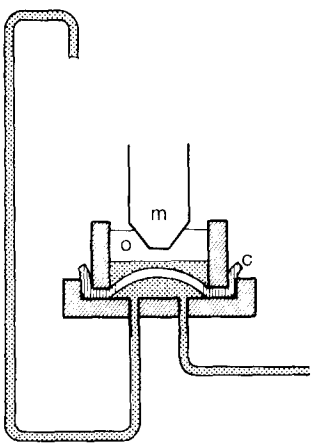

Fig. 1. Schematic diagram of the system used in these experiments. Ringer solution is perfused past the endothelial surface of the partially destromalised cornea (c). A small volume of Ringer solution is pipetted on to the bare stromal surface and is itself covered by a layer of silicone oil (o). The oil-Ringer and Ringer-stroma interfaces and the endothelium are viewed with the specular microscope, the objective of which (m) is immersed in the silicone oil.

at the beginning and end of the experiment. All samples were quantitatively analysed on a Beckman Cl/CO_2 Analyser. Total CO_2 measurements had to be corrected by a factor of 1.16 due to the effect of the buffering capacity of the Ringer on the automated acidification procedure involved in total CO_2 analysis.

Ringer samples were taken during the experiments by introducing the pipette tip into the Ringer layer through the covering layer of oil. Control experiments using an inert prosthesis in place of the corneal preparation showed that the analysis of 10-μl samples taken in this way from under oil was less efficient (12% less for total CO_2 and 6% less for Cl^-) than that of samples taken from solutions open to the air. In the same control experiments, the error on the mean of a series of measurements of 10-μl samples taken under air (1.4% for total CO_2 and 0.8% for Cl^-) increase when samples were taken under oil (2.8% for total CO_2 and 1.3% for Cl^-). Taking samples under oil from Ringer overlying an actively-pumping cornea mounted under the specular microscope did not increase the errors.

Changes in thickness of the Ringer layer were translated into volume changes by noting the thickness change associated with taking a 10-μl Ringer sample. It was demonstrated by a further experiment that such thickness changes in the overlying Ringer layer were directly proportional to the volume of translocated fluid. The perfusion outlet leading from the chamber was connected to the barrel of a 10-μl Hamilton syringe whilst the perfusion inlet was occluded. The endothelium continued to translocate fluid and to cause a reduction in the thickness of the Ringer layer. The volume of the translocated fluid could be measured directly by the movement of the outlet meniscus in the Hamilton syringe barrel and was found to be linearly related to the measured thickness change of the Ringer layer. Maurice [5] carried out a similar experiment and obtained a similar result. It has been noted in the present experiments and by Maurice [5] that the measured thickness change per unit volume in the Ringer layer was approximately twice the thickness change expected on the basis of dimensional calculations. This finding was discussed by Maurice [5] who explained the behaviour of the oil-Ringer interface as being 'between that of a membrane clamped at its edges and that of a plunger sliding in a cylinder'.

A further four experiments were carried out to determine if there was a time lag between pipetting fresh pre-warmed Ringer solution on to the stromal surface and the onset of fluid transport out of that Ringer. The accuracy with which any such time lag could be measured is limited by the accuracy of the specular microscope measurements, which are reproducible to within 2 μm.

Results

The specular microscope was used to measure the thickness of the corneal stroma and of the Ringer layer which overlay it. Fig. 2 gives a result typical of such measurements. The thickness of the Ringer layer begins to decrease whilst the stromal thickness remains relatively unchanged. Fluid is being actively translocated from the Ringer layer on the anterior stromal surface across the endothelium into the perfusing Ringer solution [5] against a hydrostatic pressure of 12 cm water.

The experiment proper begins when fresh pre-warmed Ringer solution is

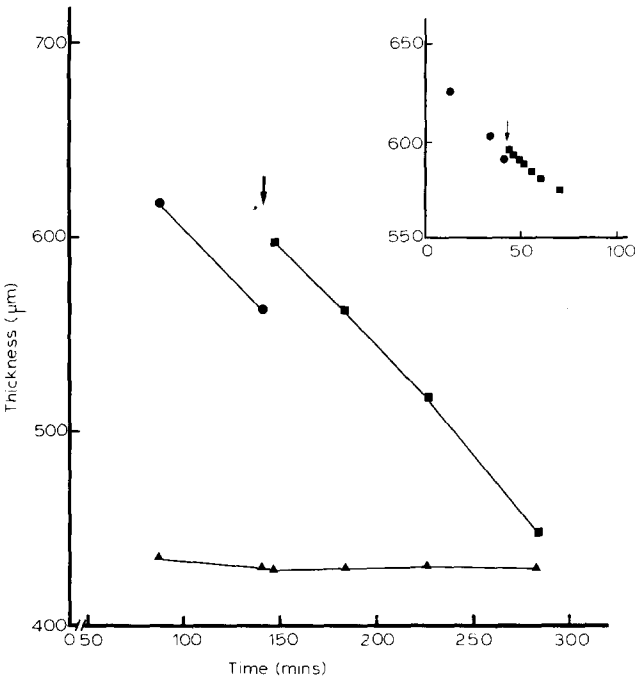

Fig. 2. Typical specular microscope result of monitoring thickness of partially destromalised corneas with overlying Ringer layer. Corneal thickness remains relatively unchanging (▲) whilst Ringer layer thickness decreases (●) showing that fluid trans-location is occurring. When the original Ringer layer is replaced (arrow) by fresh pre-warmed Ringer (■) fluid translocation continues. Inset: Typical result of experiment to find time taken for onset of fluid translocation. Arrow indicates replacement of original Ringer (●) with fresh pre-warmed Ringer (■). Corneal thickness (not shown) remained unchanging.

pipetted on to the anterior stromal surface in place of the original Ringer. The onset of fluid transfer from the fresh Ringer was noted within 3 min. In the four experiments carried out to determine the time lag, thinning of the Ringer layer was noted at 2, 2.5, 3 and 2.5 min (inset, Fig. 2). The mean rate of fluid transfer, which did not change in any consistent fashion over the whole period of the experiments, was $3.2\,\mu l \cdot cm^{-2} \cdot h^{-1}$ (range 1.9—$6.7\,\mu l \cdot cm^{-2} \cdot h^{-1}$, $n = 7$). Whilst active translocation of fluid was occurring, samples of Ringer were taken for measurement of total CO_2 and Cl^- content. In the particular experiment illustrated in Fig. 2, no samples were taken from the Ringer layer whose thickness decreased linearly. In three experiments, samples were taken throughout and the results are shown in Fig. 3. With actively pumping corneas, the total CO_2 concentration in the Ringer layer falls gradually with time (slope of regression line is significant, $P < 0.01$). The Cl^- concentration in the Ringer layer does not change (slope of regression not significant). The inset of Fig. 3 shows that the ratio of total CO_2 concentration to Cl^- concentration in each Ringer sample decreases with time in each experiment. The overall regression of the ratio of total CO_2 concentration to Cl^- concentration on time (not shown in Fig. 3) is highly significant ($P < 0.01$).

In a further four experiments, samples were taken from the Ringer layer for anion analyses at the beginning and at the end of the experiment only. The

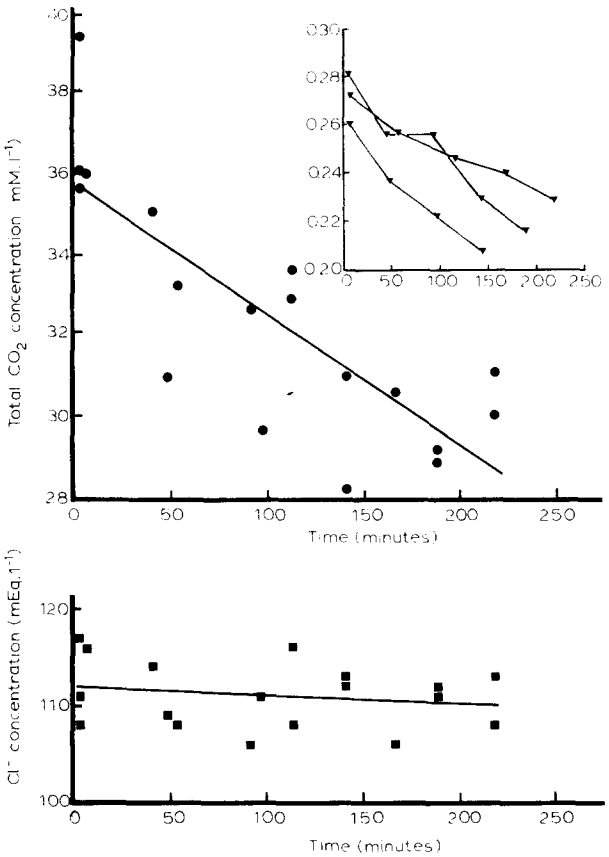

Fig. 3. Quantitative analysis of total CO_2 concentration (•) and chloride concentration (▪) in Ringer overlying actively pumping corneas. The regression of bicarbonate concentration on time is significant ($P < 0.01$). The regression of chloride on time is not significant. Inset: The ratio [total CO_2] : [Cl^-] (▲) is plotted against time in minutes. The ratio decreases with time in each experiment. The overall regression of [total CO_2] : [Cl^-] on time (not shown) is significant ($P < 0.01$).

mean net flux of total CO_2 out of the Ringer layer was $2.07 \pm 0.24 \cdot 10^{-10}$ mol $\cdot$ $cm^{-2} \cdot s^{-1}$ (± S.E.M., $n = 7$). In each of the seven experiments where Ringer analyses were carried out, both the total volume of translocated fluid and the total mass of translocated HCO_3^- is known. The concentration of total CO_2 in the exudate can be calculated in each case, giving a mean value of 0.28 ± 0.04 M ($n = 7$). The initial concentration of total CO_2 in the Ringer was 0.037 mol/l (total anion concentration, 0.156 mol/l). The exudate passing across the corneal preparation was therefore hypertonic to the bathing Ringer solution.

Discussion

Hodson and Miller [6] found an active, electrogenic HCO_3^- net flux of $1.87 \pm 0.12 \cdot 10^{-10}$ equiv. $\cdot$ $cm^{-2} \cdot s^{-1}$ across the rabbit corneal endothelium. This figure is not significantly different from the flux of total CO_2 ($2.07 \pm 0.24 \cdot$

10^{-10} mol · cm^{-2} · s^{-1}) found across similar preparations in the present experiments. Clearly, the total CO_2 flux can be largely accounted for by the net flux of HCO_3^-.

The present experiments show that if rabbit corneal endothelium, supported by about one-half the normal thickness of stromal connective tissue, is bathed in a physiological Ringer buffer, then there is a flux of HCO_3^- from stroma to aqueous which is accompanied by a flux of water in the same direction. The mean rate of fluid flux (3.2 μl · cm^{-2} · h^{-1}, range 1.9 to 6.7 μl · cm^{-2} · h^{-1}) is lower than the values of Maurice [5], obtained with the present technique and of Fischbarg et al. [11], obtained with a different technique (5.0 and 4.5 μl · cm^{-2} · h^{-1}, respectively). There is a marked variability in the fluid flux rates found in the present experiments with partially destromalised corneas which Maurice [5] and Fischbarg et al. [11] also show. This behaviour contrasts greatly with the uniform rates of deturgescence (i.e., of fluid translocation) achieved when intact but swollen corneas thin to their physiological equilibrium thickness under the influence of the same endothelial pump mechanism [10].

The system under investigation can be considered to be two bulk-phase Ringer solutions separated by a complex membrane or compartment, consisting of the endothelium and stroma plus the unstirred layers [12] adjacent to the cornea. As the bicarbonate pump of the endothelium becomes activated, HCO_3^- is translocated from one bulk-phase solution to the other, resulting in an osmotic pressure difference across the preparation. It is shown below that this bulk-phase osmotic pressure difference cannot account for the measured fluid flux across the cornea.

When fresh Ringer solution is pipetted on to the anterior stromal surface of the equilibrated preparation, linear bulk flow is established within 3 min. This time period may not represent a true lag in the onset of fluid translocation, since with the fluid translocation rates found, a thickness change would only be measurable in 2 min, due to the observational error of the specular microscope. Even so, suppose that 3 min does represent a lag phase in the response of the mechanism creating the linear bulk flow. In 3 min, $6.0 \cdot 10^{-8}$ equiv. HCO_3^- are translocated across the cornea (mean surface area 1.61 cm^2, at a rate of $2.07 \cdot 10^{-10}$ equiv. · cm^{-2} · s^{-1}). This mass, removed from the 400 μl Ringer solution overlying the anterior stroma and accompanied by a cation, would give a concentration difference (Δc) across the preparation together with the unstirred layers of $3.0 \cdot 10^{-4}$ osmol/l. The corresponding osmotic pressure difference ($\Delta\pi$) is given by:

$$\Delta\pi = RT\,\Delta c \qquad (1)$$

where R is the gas constant and T is the absolute temperature. After 3 min, an osmotic pressure difference of only $7.7 \cdot 10^3$ dynes · cm^{-2} would apply across the preparation due to the translocation of HCO_3^- and any co-ions from one bulk phase solution to the other.

The volume flow across a membrane (applicable to the cornea [14,15]) can be expressed according to the theory of irreversible thermodynamics [13]:

$$J_v = L_p\,\Delta P - \sigma L_p\,\Delta\pi \qquad (2)$$

where J_v is the fluid flux and L_p is the hydraulic conductivity of the partially destromalised cornea. The value of L_p, $1.55 \cdot 10^{-11}$ cm$^3 \cdot$ s$^{-1} \cdot$ dyne^{-1} was found for corneal endothelium by Mishima and Hedbys [14] in experiments measuring thickness changes of corneas exposed to osmotic shock. These authors also give a value of $4.9 \cdot 10^{-11}$ cm$^3 \cdot$ s$^{-1} \cdot$ dyne^{-1} for L_p of (steer) stroma, making a calculated L_p of $1.18 \cdot 10^{-11}$ cm$^3 \cdot$ s$^{-1} \cdot$ dyne^{-1} for endothelium plus full thickness stroma. The L_p in the present experiments would fall within this range of values (1.18—$1.55 \cdot 10^{-11}$ cm$^3 \cdot$ s$^{-1} \cdot$ dyne^{-1}). Since Mishima and Hedbys [14] consider that the L_p values are underestimated by some 20% due to incorrect assumptions regarding solute concentrations in the stroma, the latter value, $1.55 \cdot 10^{-11}$ cm$^3 \cdot$ s$^{-1} \cdot$ dyne^{-1} may be more appropriate to the present case. The reflection coefficient σ, in the endothelium for $NaHCO_3$ is 0.59 [15]. The σ of small solutes to the stroma is zero [14]. The hydrostatic pressure, ΔP, applied to the endothelial surface of the cornea is 12 cm water or $11.8 \cdot 10^3$ dynes/cm^2. The value of osmotic pressure difference, $\Delta\pi$, between the bulk phase solutions expected to apply across the cornea after 3 min is $7.7 \cdot 10^3$ dynes/cm^2.

The net volume flow, J_v, in Eqn. 2 has two components, hydraulic flux and osmotic flux, operating in opposition to each other. Substituting the values into the equation for osmotic flux:

$$J_v^0 = -\sigma L_p \, \Delta\pi \tag{3}$$

gives a value for osmotic fluid flux after 3 min of $7.2 \cdot 10^{-8}$ cm$^3 \cdot$ cm$^{-2} \cdot$ s^{-1}. This is smaller by an order of magnitude than the measured net flux, $8.9 \cdot 10^{-7}$ cm$^3 \cdot$ cm$^{-2} \cdot$ s^{-1} ($3.2\ \mu$l $\cdot$ cm$^{-2} \cdot$ h^{-1}).

The fluid flux due to the hydrostatic pressure difference across the cornea, given by

$$J_v^h = L_p \, \Delta P \tag{4}$$

has a value $1.8 \cdot 10^{-7}$ cm$^3 \cdot$ cm$^{-2} \cdot$ s^{-1} and occurs in the opposite direction to the osmotic fluid flux ($7.2 \cdot 10^{-8}$ cm$^3 \cdot$ cm$^{-2} \cdot$ s^{-1}). The hydraulic fluid flux is 2—3 times larger than the opposing osmotic fluid flux expected after 3 min. The calculation and comparison of fluid fluxes assumes that the same pathway is used for both osmotic and hydraulic flow. Fischbarg et al. [11] have suggested that this may not be the case and that osmotic flow can occur across the endothelium via a low conductance pathway. On this basis, the calculated value for osmotic fluid flux may be overestimated and our conclusions made even more certain.

We conclude that after an interval of 3 min the bulk phase of the fresh Ringer solution could not have been made sufficiently hypotonic, by the removal from it of HCO_3^-, for bulk phase osmosis to account for the measured trans-corneal fluid flux. The ion-water coupling must therefore be occurring within the compartment separating the two bulk solutions, i.e. within the cornea plus associated unstirred layers. The double membrane model of Curran [16] provides for such coupling, as does the standing-gradient osmotic flow model of Diamond and Bossert [17]. Both these models involve the active establishment of a local osmotic gradient into a central compartment separating two solutions (so-called 'local osmosis'). In the case of the double membrane

model, the central compartment is bound by two membranes with different reflection coefficients and contains a local high concentration of solute. Water, moving osmotically into the compartment through the first membrane, is driven out through the second membrane by a build up of hydrostatic pressure inside the compartment [18].

The standing-gradient osmotic flow model postulates that the central compartment is the intercellular space, closed at one end and open at the other. Solute pumps operate at the closed end, and create, at equilibrium, a standing-osmotic gradient down the intercellular channel. A major piece of evidence for this model is the opening of the intercellular spaces of pumping gall bladder as seen on the electron microscope [19].

The site and nature of the 'local osmotic coupling' in the partially destromalised cornea is unclear at present. Hypertonic fluid is translocated across the cornea and this is compatible with the models of ion-water coupling. From preliminary experiments it seems that the hypertonicity of the exuded fluid eventually establishes conditions where bulk osmotic flow does occur. How this could interact with the local osmosis is the subject of further investigation.

Acknowledgements

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