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The absence of bicarbonate-stimulated ATPase activity in the plasma membranes of the bicarbonate secreting ox corneal endothelial cells

Stuart Hodson and Gail Hodson

Laboratory of Experimental Optometry, University of Wales Institute of Science and Technology, Cardiff (U.K.) and Welsh National School of Medicine, Unit for Electron Microscopy, Cardiff (U.K.).

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Membranes of ox corneal endothelial cells were studied for their surface area by electron microscopical stereology and then separated from homogenates on self forming Percoll gradients. Enzymic analysis of the plasma membrane-enriched fraction failed to demonstrate an ATPase activity which was stimulated by the presence of bicarbonate ions. It is proposed that the well-established bicarbonate secretion of these cells may be coupled to the plasma membrane ($Na^+ + K^+$)-ATPase activity by the stoichiometry of Na^+/HCO_3^- close to 1:1.

Introduction

The hydration of the cornea is maintained by metabolic activity of the endothelial cells which continuously translocates bicarbonate ions from corneal stroma, across the endothelial cells and out into the aqueous humour [1]. The membrane events associated with this active transport of bicarbonate ions across the endothelium are unknown but have stimulated considerable efforts. For example, the discovery of a bicarbonate-stimulated ATPase activity in homogenates of endothelial cells resulted directly from such efforts [2]. The bicarbonate-stimulated ATPase turned out to be similar to the anion-stimulated ATPase found in other tissues and, in common with other tissues [3–5], was found in the mitochondrial-rich fraction when cellular homogenates were separated [6,7]. There remained, however, some interest in the possibility of a connection between an ATPase stimulated by the presence of bicarbonate and the endothelial bicarbonate 'pump' because the inhibition of the bicarbonate 'pump' and the anion-stimulated ATPase activity show similar inhibition characteristics in depleted bicarbonate and in their inhibition by thiocyanate and cyanate.

We decided to look for a different method for separating endothelial sub-cellular fractions and re-examine, as carefully as we could, any ATPase activity in a plasma membrane-rich fraction which may be stimulated by the presence of bicarbonate ions. Experiments were conducted over a period of years on a total of 20000 ox eyes. All these preparations showed consistent and reproducible enzymic activities. To summarise our chief result, we found no bicarbonate-stimulated ATPase activity in the plasma membranes of ox corneal endothelium.

Correspondence: S. Hodson, Laboratory of Experimental Optometry, University of Wales Institute of Science and Technology, Column Road, Cardiff, CF1 3EU, U.K.

Materials

Ox eyes were collected from the abbatoir within one hour of slaughter and transported at 0–4° C to the laboratory. Preliminary experiments were made on eyes stored up to 3 days at 0–4° C but all chief results, reported here, are from investigations conducted on the same day as slaughter. Typically, in eyes stores at 0–4° C, enzyme activities diminished at about 5% per day. The cornea was dissected as follows. The ox eye was gripped by the surrounding musculature and a small incision was made through the sclere about 3 mm from the limbus. The cornea and scleral ring were dissected with scissors, and with the cornea uppermost, the preparation was peeled off the eye taking care not to touch the cornea with the iris. The endothelial cells were isolated as follows.

Part of the scleral rim and cornea were sliced off the preparation. The remaining preparation was then shaken for 15 s in 250 mM sorbitol and then drained for 4 s by holding the free edge of the corena against filter paper. The preparation was then quickly laid on a plastic sheet, epithelium down, and most of the endothelial cells were wiped off Descemet's membrane with a single stroke of a plastic spatula pulled gently across the surface. The cells were quickly washed from the plastic spatula by rapid vibration in 250 mM sorbitol. Typically, about 3.5 cm^2 of endothelial cells were removed from each preparation. The sheets of cells isolated from ox corneas in this way retained their osmotic responsivity.

Methods and Results

Corneal endothelial cell density

Ox corneas were dissected and immersed in 5% Triton X-100 for 3 min and then fixed in 10% formal saline for 20 min. Fixative was removed with a wash in 0.9% saline. Endothelial cells were stained at room temperature by immersion for 2 min in 1 : 1 May Grunewald stain (Difco Laboratories) to water and then for 2 min in 1 : 10 Giemsa solution (Raymond Lamb) to water. The corneas were then washed free of stain in 0.9% saline and about 7-mm squares were dissected from the central cornea, laid flat on a microscope slide and photographed. cell densities were recorded against a calibrated graticule. Average endothelial cell densities of 20 ox corneas were 2076 $\pm$ 137 cell $\cdot$ mm^{-2} (mean $\pm$ S.D.). Ox corneal endothelial cells are, therefore, larger than normal human endothelial cells which typicall [8] have a density of about 2600 cell $\cdot$ mm^{-2}.

Total protein per endothelial cell

Endothelial cells were scraped from individual preparations and total protein measured in the cellular homogenate. The remaining partially de-endothelialised preparation was fixed in 10% formal saline for 2 h and then stained in 1% neutral red for 15 min. De-endothelialisation was characterised by a bright red staining of the exposed underlying stroma. In this way, the area from which the endothelium had been removed could be readily measured and compared with the total protein recovered from the cells removed by the plastic spatula. Twenty two ox corneas were examined and their mean protein density was estimated at 43.8 $\pm$ 0.9 μg protein $\cdot$ cm^{-2} (mean $\pm$ S.E.). Combining this figure with the cellular density of the previous section indicates that, on average, each ox corneal endothelial cell has about 0.21 ng protein. As the thickness of these cells is thought to be about 5 μm, then it can be estimated that the volume of each cell is 2.0 pl. Assuming the density of these cells to be about 1, ox corneal endothelial cells seem to be about 10.5% protein by weight.

Stereology of ox corneal endothelial cells

Ox corneas were dissected and fixed in 1% OsO_4 in 160 mM sodium cacodylate buffered at pH 7.4 for 1 h and prepared for electron microscopy as described previously [9]. Sections were cut orientated normally to the surface of the cornea. Essentially, the ultrastructure of ox corneal endothelial cells, which seem not to have been described previously, is the same as that of rabbit and man (Fig. 1). The paracellular space is a long fluid channel dotted with gap junctions and terminating at the apical surface in a terminal bar. 'Hairy pits' are present on the apical and basal membranes. Their function is unknown. For stereological analysis of the cells, which showed a high degree of anisotropy we choose the curvilinear grid proposed by Merz [10] and undertook dot

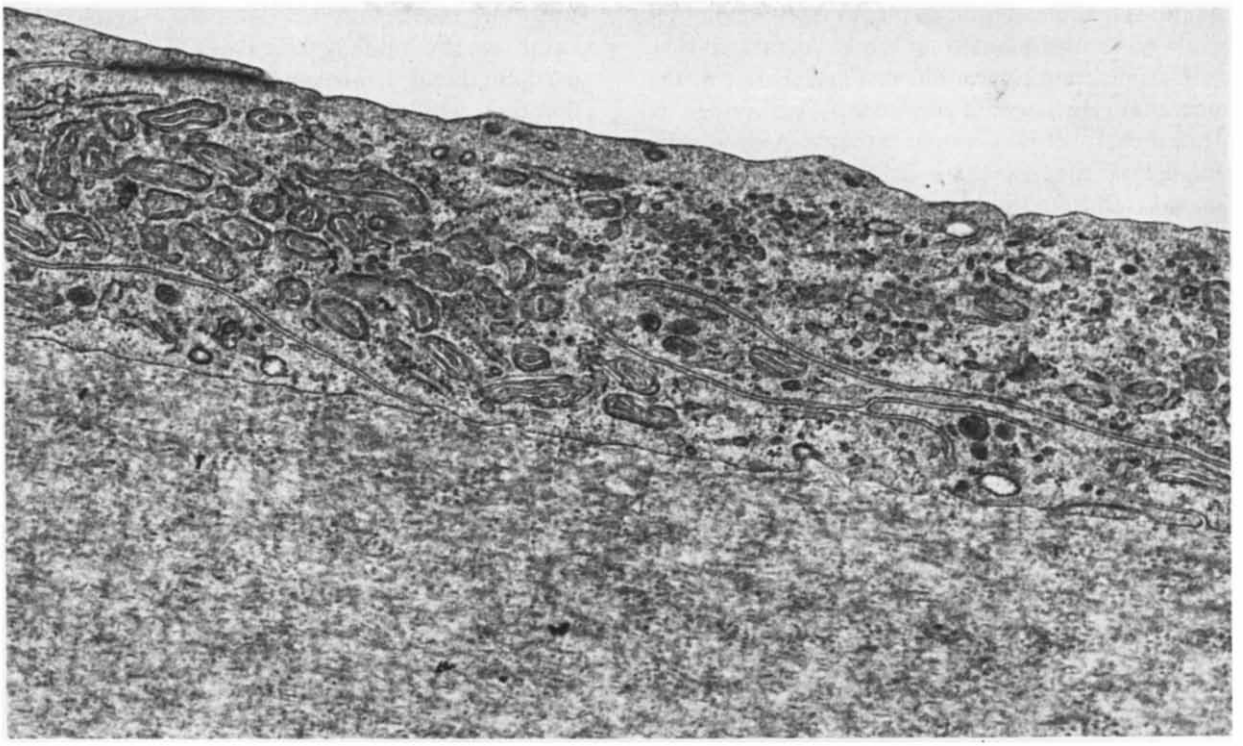

Fig. 1. The endothelium of ox cornea. Magnification: ×10500.

analysis and membrane inter-section analysis described by Weibel [11]. Random areas were chosen from eight ox corneas. In all 4225 data points on volumes, and 20545 data points on membrane surface area were noted. We believe that all our data from these eight ox eyes have an uncertainty of about 1% [11]. The data are presented in Table I.

Most of the cell is occupied by the soluble cytoplasm and the single nucleus. The remainder is occupied by the numerous mitochondria with their characteristic crystae [12]. Although rough endoplasmic reticulum and Golgi apparatus are present in these cells they contribute a negligible fraction to total cellular volume or membrane area. More than half the membrane area consists of mitochondrial membrane (55%) whereas only about 12% is nuclear. The slightly convex surface

TABLE I

STEREOLOGICAL ANALYSIS OF OX CORNEAL ENDOTHELIAL CELLS BY VOLUME AND SURFACE MEMBRANE AREA

	Cytoplasm	Nucleus	Mitochondria	Plasma membrane		
				basal	lateral	apical
Volume fraction [a]	0.647	0.183	0.169	–	–	–
Membrane area [b]	–	1.31 [c]	5.89 [c]	1.00	1.36	1.06

[a] Calculated from 4225 observations.

[b] Calculated from 20545 observations and normalised to basal plasma membrane which lies flat on Descemet's membrane.

[c] Includes both inner and outer membranes.

of the apical membrane results in it having marginally more membrane than the basal surface. The lateral membrane area allows a calculation of the maximum thickness of these cells. The average ox endothelial cell is a regular hexagon with a basal membrane area of 482 ± 72 (S.D.) μm^2 ($n = 24$) (calculated from the endothelial cell density). Each side of the hexagon is, therefore, 13.6 μm, and, from the observation that the lateral membrane occupies 1.36 more area than the basal membrane, the length of the lateral membrane is 8 μm. This is the maximum height of the endothelial cells. As the lateral margins of the cells are convoluted (Fig. 1) than it seems reasonable to assume an actual cell thickness of less than 8 μm. 'Direct' measurements of cell thickness from electron micrographs such as Fig. 1 are certain to be erroneous because of the volume changes associated with chemical fixation [9].

Sub-cellular fractionation

Ox eyes were dissected, washed vigorously in 250 mM sorbitol at 0–4°C for 15 s, drained and then the endothelial cells were scraped off Descemet's membrane, as described above, with a plastic spatula and vibrated off the spatula into 250 mM sorbitol to a final concentration of one corneal homogenate per 500 μl of solution. The cells were then homogenised with two strokes of a tight fitting Dounce homogeniser in the cold. Samples were examined under phase contrast optics. Typically very few whole cells were visible after homogenisation. Between 99 and 100% of the cells were broken. Nuclear density in the homogenate equalled original cell density before homogenisation, indicating very little breakage of nuclei. The homogenate consisted of nuclei, refractile sheets of membrane and small dark particles of size of the order of 1 μm. Samples of the homogenate were taken for analysis and of the remainder, 2 ml of suspension were overlaid on a 5 ml Percoll (Pharmacia) column in a centrifuge tube. The Percoll had previously been diluted 1 part to 7 parts of 286 mM sorbitol and had a density of 1.04 and a refractive index of 1.347. The tubes were spun at $60\,000 \times g_{av}$ and $80\,000 \times g_{max}$ for 30 min in a fixed angle rotor.

At termination, when viewed in transverse white light against a dark background, three light scattering bands were visible in the Percoll gradient. From the top they were Band 1, a nuclear rich fraction; Band 2, a plasma membrane rich fraction, and Band 3, a mitochondrial rich fraction.

If salt was present in the Percoll column, the membranes aggregated into clumps and no separation was possible. It is speculated that only in salt free aqueous solution is the natural surface charge of the membrane able to exercise sufficient inter-particulate repulsion to overcome Van der Waal's forces of attraction between the particles in low viscosity media subject to high 'g' forces.

Enzyme assays

Succinate–cytochrome-*c* reductase was assayed by the method of Mahler [13].

ATPase was assayed by one of two methods. Our most commonly used method was that of continuous assey of ADP release where we recorded rate of change in absorbance at 340 nm caused by the oxidation of NADH in the incubation mixture as pyruvate is produced by the liberated ADP combining with phospho*enol*-pyruvate [14]. The incubation mixture included additionally a final concentration of 25 mM buffer, usually Tris-HCl, at pH 7.6, 100 mM NaCl, 5 mM KCl, 1 mM EGTA and to block $(Na^+ + K^+)$-ATPase activity as necessary, 10^{-4} M ouabain. To examine Ca^{2+}-ATPase activity, EGTA was omitted from the cuvette and 1 mM $CaCl_2$ added. The cuvettes were allowed to stabilise for 10 min at 30°C in the spectrophotometer. (The system was not stable at physiological temperatures.) After stabilising absorbance at 340 nm, 100 μl of particulate fraction was added to the incubation volume of 2.1 ml and rapidly mixed. Changes in absorbance at 340 nm were monitored simultaneously in four cuvettes for at least 40 minutes.

In the second method of measuring ATPase activity, liberated phosphate was measured by the method of Lebel, Poirier and Beaudoin [15]. Both methods gave similar values of ATPase activity, their main difference was that the continuous spectrophotometric assay gave better reproducibility.

All enzymic activities reported in this paper were linearly related to amount of particulate fraction added in the activity ranges recorded.

Table II records typical results for particulate

TABLE II

PERCOLL FRACTIONATION OF OX CORNEAL ENDOTHELIAL CELLS POOLED FROM FOUR CORNEAS

Figures presented are averages ± S.D. of twelve determinations. Figures in parentheses indicate percentage recovery. n.d., not detected.

Fraction	Total protein (mg)	Cytochrome oxidase (mmol/mg protein per h) [a]	Basal ATPase (μmol/mg protein per h)	$(Na^+ + K^+)$-ATPase	
				μmol/mg protein per h	μmol/cm² per h
Homogenate	614 ±19 (100)	0.63 (100)	1.87 ± 0.08 (100)	7.64 ± 0.20 (100)	0.335 [b]
Supernatant	335 ±23 (58)	n.d.	0.28 ± 0.07 (9)	0.26 ± 0.06 (2)	0.011
Band 1, nuclear-enriched fraction	93.4 ± 8.4 (15)	n.d.	1.48 ± 0.25 (12)	8.73 ± 1.24 (17)	0.380
Band 2, plasma membrane-enriched fraction	129.1 ± 8.8 (21)	0.285 (10)	1.52 ± 0.16 (17)	19.32 ± 0.49 (53)	0.840
Band 3, mitochondria-enriched fraction	121.5 ± 13.1 (20)	2.27 (71)	3.38 ± 0.27 (36)	0.79 ± 0.19 (2)	0.034

[a] Errors on cytochrome oxidase activity ± 5%.
[b] Calculated as 43.5 μg protein/cm² of endothelium.

fractions assayed for ATP-ase in a final concentration of 25 mM Tris-HCl buffered at pH 7.6, 100 mM NaCl, 5 mM KCl, 1 mM EGTA and, to record basal ATP-ase activity, with 10^{-4} M ouabain.

The absence of a buffer in the cell homogenisation medium did not affect ATPase activity as enzyme activities were essentially unchanged in the presence or absence of buffer during homogenisation. Anion-sensitive ATPase activity was not found to be spontaneously present in our fractions. Only when we froze and thawed the fractions did the activity appear as a cyanate inhibitable activity associated with the mitochondrial fraction, as reported previously [2,6]. In our hands, some disruption of mitochondrial membranes must precede the appearance of the anion-sensitive ATPase activity.

We looked for spontaneous bicarbonate ATPase activity in all fractions by the following strategy.

We incubated in sealed cuvettes including 5, 10, 20 or 40 mM bicarbonate buffered at pH 7.0–8.2 in steps of 0.2, either by Tris-HCl or Hepes. Incubation mixture pH was monitored both before and after incubation and, once the cuvettes were satisfactorily sealed, remained constant.

The effects of $[Na^+]$, 0, 20, 40, 60, 80 or 100 mM or of $[K^+]$, 0, 5, 10, 50 or 100 mM or of $[Ca^{2+}]$, 0, 0.1, 0.5, 1, 5 mM, or of the anions chloride, sulphate, nitrate and phosphate were examined. In no case was there any detectable HCO_3^--stimulated ATPase activity in any fraction. Potential co-factors were added: 0.1, 0.5, 1 or 5 mM reduced or oxidised glutathione; 1 or 5 mM dithiothreitol. Their addition did not demonstrate any bicarbonate-stimulated activity. We noted an activity of Ca^{2+} stimulated-ATPase activity of 0.9 ± 0.2 μmol/mg protein per h in the homogenate, assayed at 30 °C (pH 7.6). However, we found no bicarbonate-stimulated ATPase activity in the plasma membrane-enriched fraction of ox corneal endothelial cells.

Discussion

Although we are unable to report any bicarbonate-stimulated ATPase activity in the plasma membranes of ox corneal endothelial cells, we are, of course, not able to discount the possibility of such an activity which might appear under different assay conditions. In all we made several thousand attempts to detect an activity, most of which are not systematic and are not reported here. Some other interesting results arose incidental to our investigation.

First, we found a rapid method for separating quite efficiently the major sub-cellular functions of these cells, using Percoll self-forming gradients.

A potentially interesting observation is that the plasma membrane-enriched fraction had a fine structure and seemed to consist of two bands of only slightly different densities. Possibly, this may represent a separation of baso-lateral and apical plasma membranes.

Second, we report here higher activity of $(Na^+ + K^+)$-ATPase in homogenates of these cells than has been reported elsewhere [6,7]. The significance of this, if one follows the arguments on the quantitative aspects of the energetics of endothelial transport developed by Riley [16] is that $(N^+ + K^+)$-ATPase activity of 0.335 $\mu mol \cdot cm^{-2} \cdot h^{-1}$ allows for a maximal *trans*-membrane transport of sodium of 1 $\mu equiv. \cdot cm^{-2} \cdot h^{-1}$ which is now seen to be near to that trans-endothelial transport rate of bicarbonate. There is no measurable net flux of sodium across corneal endothelium under short circuit [17]. We propose, from the current data, that there is a coupling between *trans*-membrane active sodium flux and *trans*-endothelial net bicarbonate flux and that the stoichiometry could be Na^+/HCO_3^- 1:1. The membrane mechanisms associated with this coupling await discovery.

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