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THE LOCALIZATION OF THE ANION-SENSITIVE ATPase ACTIVITY IN CORNEAL ENDOTHELIUM

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The localization of the anion-sensitive ATPase (EC 3.6.1.3) of bovine corneal endothelium has been investigated. Homogenates were fractionated by differential and density gradient centrifugation, into fractions enriched in plasma membranes and mitochondria. ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) and cytochrome oxidase (EC 1.9.3.1) were used as marker enzymes for these two cell components, and glucose-6-phosphatase (EC 3.1.3.5) was used to identify endoplasmic reticulum. 5′-Nucleotidase (EC 3.1.3.5) was also measured but was found not to be exclusively associated with any one cell component. The activity of the anion-sensitive ATPase (HCO_3^--ATPase) was measured in suspensions that were frozen and thawed before assay in order to expose latent enzyme activity. The fraction containing the greatest amount of ($Na^+ + K^+$)-ATPase (35%) contained only 6% of the cytochrome oxidase and HCO_3^--ATPase. Conversely, the mitochondrial fraction, containing 40% of the cytochrome oxidase, contained about 40% of the HCO_3^--ATPase, but only 7% of the ($Na^+ + K^+$)-ATPase. The recoveries and relative degree of purification of the cytochrome oxidase and HCO_3^--ATPase were also nearly identical in the other fractions examined. It was concluded that the anion-sensitive ATPase activity of the corneal endothelium is located solely in the mitochondria and not in the plasma membrane. Consequently, any role that the enzyme may have in the transport of bicarbonate across this tissue, which had been suggested in earlier studies, must be an indirect one.

Introduction

Active transport of bicarbonate ion across the corneal endothelium has been demonstrated in mammalian corneas during in vitro studies and is postulated to be the driving force for fluid transport from the stroma into the aqueous humor [1,2], a process essential for maintaining transparency of the cornea [3]. It has also been shown that the endothelial cells contain an anion-sensitive ATPase that is maximally stimulated by bicarbonate and whose activity is of a magnitude which suggests it could function as a coupling system to provide the necessary energy for active transport of this ion [4]. However, while similar postulates have been made for other bicarbonate-transporting tissues, such as stomach [5], pancreas [6] and kidney [7], in no case is there conclusive evidence that a bicarbonate-stimulated ATPase drives ions across cell membranes in a manner analogous to that established for the ($Na^+ + K^+$)-ATPase. While the results of certain cell fractionation studies have indicated that the enzyme is present in the plasma membrane [7–10], several other studies have found the enzyme to be mitochondrial in origin and conclude that it has no role in transcellular bicarbonate transport [11–14]. Unfortunately, it has not been possible to use histochemical methods to distinguish between these positions for the methods suitable for other phosphatases [15] are rendered useless by the presence of bicarbonate and no inhibitor with an affinity or specificity comparable to that of ouabain has been available for binding studies [16].

We have investigated the distribution of the anion-sensitive ATPase in the corneal endothelium because of the physiological importance of the transport

of bicarbonate ion in corneal transparency. An advantage of this tissue for fractionation studies is that it is comprised of a single cell type, clearly separated from other components of the cornea by a thick basement membrane, and therefore the results should be helpful in resolving some of the uncertainties in studies of other tissues.

Methods and Materials

Bovine eyes were obtained from the slaughterhouse and immediately chilled on ice. Within 2 h the corneas were excised, drained of aqueous and scraped with a spatula to remove the endothelial cells. Descemet's membrane was not removed by the gentle scraping. The cells from 40 corneas were collected in 4.0 ml of 8.3 mM Tris-maleate buffer, pH 7.6. The suspension was filtered through a polypropylene 105 μm mesh (Spectrum Medical Industries, Los Angeles, CA) which was rinsed with a further 1.0 ml buffer, and then sonicated for 45 s in a Bransonic 12 (Branson Instruments, Shelton, CT). This suspension was designated homogenate (H) and, after removal of an aliquot for assays, it was centrifuged at $600 \times g$ for 12 min to yield a supernatant (S) and a 'nuclear' pellet (PN). The supernatant was centrifuged at $20\,000 \times g$ for 20 min to yield a mitochondrial pellet (M), and a supernatant containing soluble and microsomal material (McS).

The nuclear pellet was further fractionated by a discontinuous density gradient procedure [17]. The pellet was resuspended in 2.0 ml buffer and divided into 0.6-ml aliquots which were layered on three tubes containing 2.0 ml of 10% sucrose in 13% dextran (M_r 500 000, Sigma Chemical Co., St. Louis, MO) overlying 2.0 ml of 54% sucrose. The tubes were centrifuged at $114\,000 \times g$ for 30 min in the SW 50.1 head of a Sorvall OTD 65. After centrifugation, the upper phase (F_1) was removed from each tube and combined, followed by the interphase region (F_2) and then the lower phase (F_3). Total volumes from the three tubes were approximately 6.8, 2.0 and 4.6 ml for F_1, F_2 and F_3, respectively. The F_2 fraction was diluted with buffer to 10.0 ml and, after removal of an aliquot for assay, was centrifuged at $30\,000 \times g$ for 20 min. The pellet was resuspended in buffer, yielding the fraction designated CF_2. On two occasions F_1 and F_3 were diluted 3-fold and spun as for F_2, and the pellets were resuspended to give CF_1 and CF_3.

All fractions were diluted before assay to appropriate concentrations with 8.3 mM Tris-maleate buffer. ATPase, cytochrome oxidase and protein assays used the same final preparations, while assays for 5′-nucleotidase and glucose-6-phosphatase required more concentrated preparations because of the lower activities of these two enzymes. ATPase assays were conducted according to the methods described by Riley [4]. $(Na^+ + K^+)$-ATPase was defined as the activity inhibited by 10^{-4} M ouabain and anion-sensitive ATPase activity (which will be referred to synonomously with HCO_3^--ATPase since this is the anion of major importance for corneal function) as that elicited, in the presence of ouabain, by substitution of 25 mM $NaHCO_3$ for 25 mM NaCl. This activity was always determined on fractions which were quickly frozen in solid CO_2/acetone before assay for reasons given below. Cytochrome oxidase activity was measured at 23°C by the method of Wharton and Tzagoloff [18], 5′-nucleotidase by the method of Widnell and Unkeless [19], using acetate buffer, and glucose-6-phosphatase by the method of Hubscher and West [20]. All phosphatase assays were incubated at 37°C for 30 min and terminated with ice-cold 8% trichloroacetic acid, and the phosphate liberated was determined by the method of LeBel et al. [21]. Protein was assayed by the BioRad microprocedure (BioRad, Richmond, CA) using bovine gamma globulin as standard. All assays were carried out in duplicate, with appropriate tissue and reagent blanks.

The procedures described above were designed to elicit maximum expression and recovery of the several enzymes in the endothelium and each of its constituent fractions. Sonication was preferred to homogenization in a Dounce homogenizer because the latter procedure resulted in a lower specific activity of $(Na^+ + K^+)$-ATPase in the homogenate. The strength of the Tris-maleate buffer was determined by the conflicting needs for a sufficient ionic strength to prevent clumping of particles during centrifugation, and a medium dilute enough to permit rupture of the cells during sonication and effective freeze-thawing. Without freezing, the activity of the anion-sensitive ATPase of homogenates

in buffers or sucrose was very low, and only in distilled water could the same activity be demonstrated in frozen and non-frozen homogenates. In water homogenates or freeze-thawed preparations in dilute buffers the specific activity of the anion-sensitive enzyme was approx. 1.5-fold that of the $(Na^+ + K^+)$-ATPase, a value similar to that previously observed in water homogenates of rabbit cornea [4]. In no fraction did non-frozen exceed that of frozen activity, although the former did increase with successive fractionation steps, leading to apparent 'recoveries' of well over 100%. This requirement of freezing explains the need for washing the F fractions (yielding CF) since even after a 5-fold dilution with Tris maleate their high sucrose concentrations prevented effective freeze-thawing. Neither the sucrose nor dextran in the diluted F fractions interfered with the assay of the other enzymes examined.

Results

Table I shows the specific activities of the enzymes in each of the fractions obtained and of the initial homogenate. $(Na^+ + K^+)$-ATPase, 5′-nucleotidase and glucose-6-phosphatase were each concentrated to some extent in the PN fraction and, after fractionation on the gradient, all three enzymes showed their highest specific activity in the F_2 fraction. The $(Na^+ + K^+)$-ATPase was purified 10-fold, the other two enzymes by 3- to 4-fold. Cytochrome oxidase and HCO_3^--ATPase were also concentrated somewhat in PN, but in contrast with the pattern for the other three enzymes, their specific activities after the gradient separation were highest in the uppermost layer, F_1 (or CF_1), and in F_2 were lower than in the PN fraction. The greatest purification of these two enzymes was found in the M fraction where the specific activities were about four times higher than in the homogenate.

In addition to the relative purification of the enzymes in each fraction, the amount of total enzyme activity was also determined. Table II shows the recoveries expressed as percentages of the total activities measured in the homogenate, and also the distribution of protein in the fractions. For each of the enzymes measured, the total recovery in the first two fractions, PN and S, was more than 90% of that in the homogenate. Subsequently, 70 to 90% of the total activities in the PN fraction was recovered from the gradient (except in the case of 5′-nucleotidase), and the M and McS fractions accounted for 90 to

TABLE I

SPECIFIC ACTIVITIES OF ENZYMES AFTER FRACTIONATION OF HOMOGENATES OF CORNEAL ENDOTHELIUM

For abbreviations of fractions see Methods and Materials. Hydrolase activities expressed as μmol P_i liberated/mg protein per h. Oxidase activity expressed as mmol cytochrome *c* oxidized/mg protein per h. Values are Mean ± S.E.M. of eight experiments (excluding* where $n = 2$).

Fraction	$(Na^+ + K^+)$-ATPase	HCO_3^--ATPase	Cytochrome Oxidase	5′-Nucleotidase (X10²)	Glucose-6-phosphatase (X10²)
H	3.6 ± 0.7	5.2 ± 1.7	2.1 ± 0.3	12 ± 2	27 ± 1
PN	12.7 ± 1.0	11.7 ± 0.9	5.4 ± 0.4	19 ± 2	64 ± 7
S	0.4 ± 0.1	2.9 ± 0.3	1.1 ± 0.3	9 ± 2	17 ± 1
F_1	6.0 ± 0.7	–	5.7 ± 1.0	25 ± 3	68 ± 4
F_2	35.8 ± 4.1	–	3.9 ± 0.8	44 ± 6	69 ± 8
F_3	12.7 ± 1.4	–	2.5 ± 0.3	19 ± 4	15 ± 6
CF_1^*	11.8	14.6	7.1	–	–
CF_2	30.5 ± 5.1	7.6 ± 1.1	3.9 ± 0.9	–	–
CF_3^*	8.1	9.1	3.2	–	–
M	3.5 ± 0.5	18.7 ± 2.4	8.9 ± 1.9	27*	50*
McS	0.1 ± 0.1	0.1 ± 0.1	0 ± 0	5*	6*

TABLE II

RECOVERY OF PROTEIN AND ENZYME ACTIVITIES RELATIVE TO TOTAL ACTIVITIES IN THE HOMOGENATE

For abbreviations of fractions see Methods and Materials. Values are Means ± S.E.M. of eight experiments (excluding* where n = 2), expressed as percentages.

Fraction	Protein	$(Na^+ + K^+)$-ATPase	HCO_3^--ATPase	Cytochrome Oxidase	5′-Nucleotidase	Glucose-6-phosphatase
H	11.9 ± 1.3 mg	100	100	100	100	100
PN	22 ± 1	86 ± 2	53 ± 6	56 ± 6	39 ± 5	53 ± 1
S	75 ± 2	10 ± 2	43 ± 6	38 ± 8	56 ± 5	47 ± 1
F_2	4 ± 1	35 ± 3	–	6 ± 1	14 ± 3	13 ± 2
CF_2	3 ± 0	25 ± 3	6 ± 1	6 ± 1	–	–
M	11 ± 1	7 ± 2	38 ± 4	42 ± 7	31*	37*

100% of the activities in the supernatant (S).

While only 22% of the protein was found in the PN fraction, more than 50% of every enzyme except 5′-nucleotidase was found here, and as much as 86% of the $(Na^+ + K^+)$-ATPase. When PN was separated on the gradient, 35% of the $(Na^+ + K^+)$-ATPase activity of the homogenate could be recovered in F_2. On washing this fraction there was some loss of activity, the recovery falling by 28% (to 25% of H) while the protein lost on washing was only 14% (decreasing from 0.48 mg to 0.41 mg). The recoveries of 5′-nucleotidase and glucose-6-phosphatase in the F_2 fraction were 14% and 13% of the homogenate values respectively. Total activity of 5′-nucleotidase in F_1–F_3 was more than initially present in PN, indicating some 'activation' of this enzyme.

Only 6% of the cytochrome oxidase activity was recovered in the F_2 fraction on the gradient, and this activity remained in the washed fraction CF_2. The HCO_3^--ATPase activity, which could not be measured in F_2 because the high sucrose concentration precluded effective freezing, was also found at 6% of the homogenate value in fraction CF_2. In the two experiments where F_1 and F_3 were also centrifuged, to yield CF_1 and CF_3, the relative recovery of HCO_3^--ATPase and cytochrome oxidase was twice as high in CF_1 as in CF_2, while for $(Na^+ + K^+)$-ATPase, the reverse was true.

Approximately 40% of the cytochrome oxidase and HCO_3^--ATPase activities were recovered in the M fraction after the 400 000 $g \cdot$ min spin and only 7% of the $(Na^+ + K^+)$-ATPase activity. There was negligible activity of these enzymes in the microsomal/soluble fraction. Measurable amounts of only 5′-nucleotidase and glucose-6-phosphatase were found in this fraction.

Discussion

The determination of the intracellular localization of an enzyme by cell fractionation requires that reliable marker enzymes be known for the fractions of interest, that a high yield of these enzymes and the enzyme in question can be recovered, and that a high degree of purification can be achieved. The monitoring of total recovery is important to establish that neither inhibition nor activation of enzymes results from the techniques employed and to measure the extent of any solubilization of membrane bound enzymes that might occur.

$(Na^+ + K^+)$-ATPase has been used as a reliable marker for plasma membranes in many studies, and has been shown by histochemical methods to be present on the lateral cell membranes of the corneal endothelium [22,23]. Cytochrome oxidase is a constituent of the inner mitochondrial membrane and glucose-6-phosphatase is identified with the endoplasmic reticulum of the cell. 5′-Nucleotidase has recently been used as a cell membrane marker for corneal endothelium [24], although in liver it is not restricted to the plasma membrane, but is found in rough and smooth endoplasmic reticulum [19]. Histochemical studies of its distribution in the

corneal endothelium have not been made.

Without meeting the above criteria it is not possible to conclude with any certainty that a particular enzyme is exclusively located at any one site in the cell. Van Amelsvoort et al. [11] have noted that the decrease in ratio of succinate dehydrogenase activity to HCO_3^--ATPase in a fraction may be due to an activation of ATPase by loss of inhibitor or increased access of substrate, rather than due to elimination of mitochondrial content as suggested in some studies [7,25]. Precisely such an activation was found in the present study when the HCO_3^--ATPase of the unfrozen preparations was measured, for the activity increased at each fractionation step. Only by ensuring maximum activity in each of the fractions by freezing was this artifact eliminated. The assay of cytochrome oxidase and $(Na^+ + K^+)$-ATPase in all the isolated fractions of the tissue further ensured that no significant amounts of mitochondria or plasma membranes were overlooked. Total yield of both these enzymes in F_1-F_3 and M together was over 70%.

The 35% recovery and 10-fold purification of $(Na^+ + K^+)$-ATPase in F_2 indicates that this fraction is significantly enriched in content of plasma membranes. There are also membranes of mitochondria and endoplasmic reticulum present, as indicated by the 6% of cytochrome oxidase and 13% glucose-6-phosphatase, but the lack of purification of the enzymes shows that these components have not been concentrated in this fraction. 5′-Nucleotidase was also present in the F_2 fraction but its low recovery and its presence in the McS fraction together with glucose-6-phosphatase suggest that it is not restricted to plasma membranes alone, but is present also in endoplasmic reticulum, as found in other tissues [11,19].

Several attempts were made to eliminate the mitochondrial and endoplasmic reticulum contaminants from the F_2 fraction. The fraction was diluted, then sonicated or homogenized and resedimented on the gradient. After either mode of treatment the proportion of $(Na^+ + K^+)$-ATPase recovered was no greater than that of cytochrome oxidase and there was no increase in specific activity. Similar results were obtained when the F_2 fraction was added to and separated in the two-phase aqueous polymer system described by Lesko et al. [26]. It was concluded from these failures to remove the extraneous material that mitochondrial and endoplasmic reticulum were probably surrounded by plasma membranes in vesicle-like structures formed during homogenization. Such locking of contaminating particles into the plasma membranes was found when brush border membranes were isolated from kidney [12].

It is clear from the results that only a very small fraction of the HCO_3^--ATPase is associated with the F_2 fraction. Moreover, both the pattern of recovery and the relative degree of purification of the HCO_3^--ATPase are almost identical to those of the cytochrome oxidase throughout the entire fractionation procedure, and are in marked contrast to the distribution and purification of the $(Na^+ + K^+)$-ATPase. This parallel behavior of the cytochrome oxidase and the HCO_3^--ATPase in both the gradient fractions and the mitochondrial fraction strongly indicates that the anion-sensitive enzyme is located solely on the mitochondrial membrane, and that its activity in the plasma membrane fraction is due to contamination by mitochondria.

This study was inspired by the evidence that control of stromal hydration and fluid transport across the endothelium are dependent upon the active transport of bicarbonate ions [1,2]. Since it was also shown that there is a correlation between the fluid transport rate and HCO_3^--ATPase activity at varying bicarbonate concentrations, and a similar inhibitory effect of cyanate on the enzyme and on net fluid movement [4,27], it was anticipated that the HCO_3^--ATPase might be found on the plasma membrane where it could function as a bicarbonate pump. This is clearly not the case. Other studies have also found the anion-sensitive ATPase to be of mitochondrial origin and have concluded that the enzyme is not involved in the active transport of bicarbonate across the plasma membrane [11–14, 28,29]. However, in view of the apparent relation in the cornea of fluid movement and HCO_3^--ATPase activity, it does seem possible that it may have some secondary role in bicarbonate transport. The primary system of ion transport in the cornea remains elusive, there being no evidence to date for a system like the proton pump and $(H^+ + K^+)$-ATPase of the gastric mucosa [30,31], and there being significant difficulties in reconciling physiological data with the operation of a sodium pump and the $(Na^+ + K^+)$-ATPase [1,27,32].

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