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Amphotericin B enhanced anomalous potential difference response to changes in aqueous K^+ in frog cornea

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An increase in aqueous K^+ from 0 to 4 mM increased the potential difference (anomalous response of electrogenic $(Na^+ + K^+)$-ATPase antiport) by 1.1 mV in Cl^--free solutions compared to 6.8 mV in Cl^- solutions. With amphotericin B added to the tear solution in Cl^--free solutions, the anomalous PD response for the addition of 4 mM K^+ to the aqueous solution was about 20 mV, significantly greater than in Cl^- solutions. This anomalous response was inhibited by ouabain. These data support the electrogenicity of the $(Na^+ + K^+)$-ATPase pump. It is also evident that, for the pump to respond, Na^+ should readily enter the cell. This may be accomplished experimentally, either across the basolateral membrane in Cl^- solutions or across the apical membrane in Cl^--free solutions with amphotericin B present in the tear solution.

Introduction

When frog cornea was bathed in K^+-free solutions for more than 5–10 min, addition of 4 mM K^+ to the stroma solution resulted in an increase in potential difference. We have called this increase in PD an anomalous response because it is opposite to that expected for a simple K^+ conductive pathway [1]. We have attributed this anomalous response to electrogenic $(Na^+ + K^+)$-ATPase located in the basolateral membrane of the cornea epithelial cells transporting more Na^+ ions than K^+ ions per cycle. A similar finding was previously described in the frog gastric mucosa [2]. While the anomalous response was reproducibly eliminated by ouabain in the frog stomach experiments, in the cornea ouabain decreased markedly the anomalous response in all experiments, but complete elimination of the response was obtained only in about half of the experiments.

The ambiguous effects of ouabain threw doubt on the $(Na^+ + K^+)$-ATPase's being solely responsible for the anomalous PD response. An electrogenic passive NaCl symport has been found responsible in the frog gastric mucosa for anomalous PD responses obtained to changes in Na^+ concentration in the nutrient solution [3,4]. More Cl^- than Na^+ ions are assumed to be transported per cycle through that symport. Perhaps a pathway involving K^+, such as a passive KCl symport, transporting more Cl^- than K^+ ions per cycle, could be responsible for the minimal anomalous responses observed in the presence of ouabain.

In experiments to be presented in this paper it will be shown that removal of Cl^- from the bathing solutions diminished or abolished the anomalous PD response to changes in K^+ concentration. But when amphotericin B was added to the tear solution, a marked anomalous response was obtained in the absence of Cl^-. This response was

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completely abolished by ouabain. Candia et al. [5,6] have shown a marked increase in the net Na^+ flux from tear to stroma and an increase in the net K^+ flux from stroma to tear when amphotericin B was added to the tear solution in Cl^--free solutions. They attributed the response to an increase of the apical membrane conductance to Na^+ and K^+ by amphotericin B.

Methods

Experiments were performed on corneas of the bullfrog (*Rana catesbeiana*) by an in vitro method in which the corneas were mounted between a pair of cylindrical chambers [1,7,8].

All experiments were begun with physiological (control) solutions on both sides of the cornea. Control solutions contained in mM: Na^+ 102, K^+ 4, Ca^{2+} 1, Mg^{2+} 0.8, Cl^- 81, SO_4^{2-} 0.8, HCO_3^- 25, phosphate 1, and glucose 10. Na^+ was substituted for K^+ in K^+-free solutions. SO_4^{2-} was substituted for Cl^- in Cl^--free solutions with appropriate addition of sucrose for osmolality correction. Amphotericin B was added to the tear solution to a final concentration of $1 \cdot 10^{-5}$ M. Ouabain was added to the aqueous solution to a final concentration of $1 \cdot 10^{-4}$ M.

Both sides of the cornea were continually gassed with 95% O_2/5% CO_2. Gassing was used also to recirculate and stir thoroughly the solutions. The pH of the solution was 7.2–7.3.

To avoid transmembrane changes in pressure when changing the solutions, the chambers were never emptied. The new solution was injected at a high flow rate (10- to 15-times the volume of the chamber per minute) from the bottom of the chamber. The outflow was located on the top of the chamber. The outflow tube of the aqueous solution was located about 15 cm in about one-third of the experiments and 2 cm in about two-thirds of the experiments, above the outflow tube of the tear solution. The 15 cm pressure difference was used because of its similarity to the in vitro intraocular pressure. The 2 cm pressure difference was sufficient to preserve the natural shape of the cornea. The electrical parameters were not affected by a change from one pressure gradient to the other.

The transepithelial resistance (R) and transepithelial electrical potential (PD) were measured. Two pairs of electrodes were used: one for sending current across the mucosa and the other for measuring the PD. The PD was taken as positive when the aqueous side was positive to the tear side. The resistance was determined as the change in PD per unit of applied current. 2 μA were applied for 1 or 2 s in one direction and 2 or 3 s later in the other direction. No significant rectification was observed. Student's t-test using paired observations was used to determine the level of significance, when applicable.

Results

Effect of removing Cl^- from bathing solutions

Fig. 1 shows that removal of Cl^- from the aqueous solution resulted in a decrease in PD of about 15 mV. Upon removal of Cl^- from the tear solution the PD increased slightly.

Table I shows data from 14 experiments in which Cl^- was removed from the aqueous solution and from 9 experiments in which Cl^- was removed from the tear solution. Removal of Cl^- from the aqueous solution resulted in a significant decrease in PD and an increase in resistance. Removal of Cl^- from the tear solution resulted in significant increases in PD and resistance.

Effect of removal of Cl^- on the anomalous PD response to change in K^+ concentration

As indicated in Fig. 1, at about the 30 min mark, a typical anomalous PD response [1] occurred when K^+ was increased from zero to 4 mM in the aqueous solution, in the presence of Cl^-; that is, the PD increased by about 5–6 mV, contrary to that expected for a simple K^+ conductance pathway in the basolateral membrane (see Introduction). In the absence of Cl^-, minimal PD changes were observed when the K^+ was changed from 4 mM to zero or back to 4 mM in the aqueous solution. The top two rows of Table II show data from 14 experiments in which K^+ was removed and returned to 4 mM in the aqueous solution in Cl^--free solutions. Removal of K^+ resulted in a small but significant increase in PD. The maximum increase was 2.2 mV, remaining at 1.0 mV 10 min after the change in concentration. There was a small but significant increase in R.

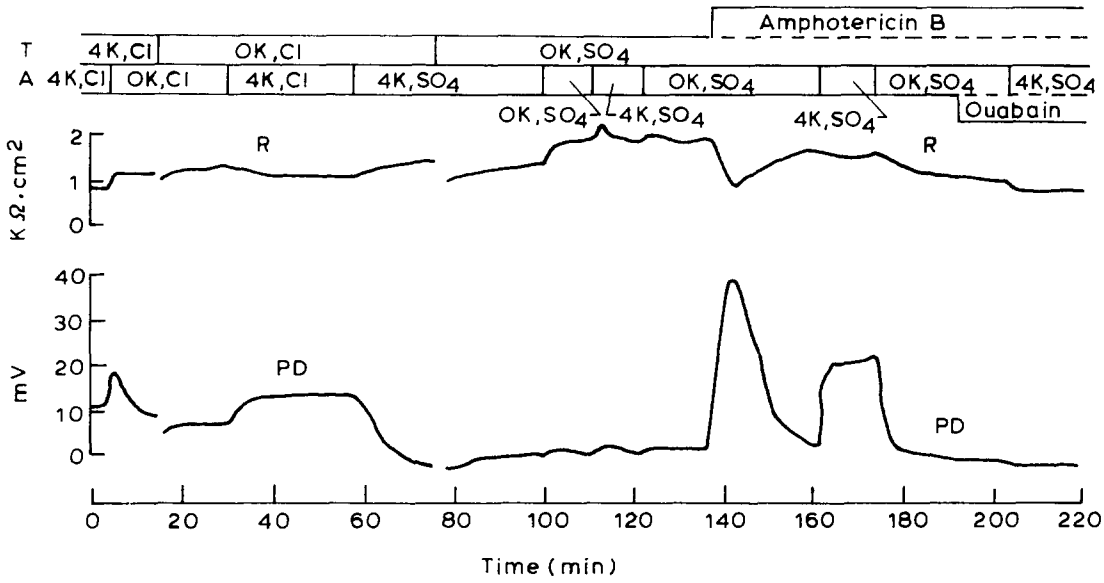

Fig. 1. Effect of removal of Cl^-, and then addition of 10^{-5} M amphotericin B in the tear solution, followed by 10^{-4} M ouabain in the aqueous solution on the PD and resistance changes induced by changing K^+ in the aqueous solution to 0 and back to 4 mM.

When $[K^+]$ was changed back to 4 mM the PD initially increased (anomalously), reaching a maximum of 1.1 mV, but at 10 min the PD had significantly decreased by 0.8 mV below control. The decrease in PD is a normal response and is contrary to what is observed in the presence of Cl^- [1]. Ouabain was added to the aqueous solution to a concentration of about 10^{-4} M, without any change in PD or resistance. In the presence of 10^{-4} M ouabain in the aqueous solution the initial anomalous response was not observed. Only the normal decrease in PD was seen, which, although small, was significant. No significant change in resistance was observed when K^+ was changed in the presence of ouabain.

We consider the possibility that the initial minimal and short-lasting anomalous PD response, when K^+ is increased from 0 to 4 mM in Cl^--free solutions, is due to the low concentration of Na^+ in the cells which prevents a proper response of the $(Na^+ + K^+)$-ATPase pump. If the small anomalous PD response is due to the low

TABLE I

EFFECT OF CHANGING Cl^- FROM 81 TO 0 mM (SO_4^{2-} SUBSTITUTED) FROM BATHING SOLUTIONS IN THE FROG CORNEA

Values are means ± S.E. PD and R values obtained before change. Δ(max) values obtained at highest PD following change (< 10 min). Δ(10) obtained 10 min after change.

mV			$k\Omega \cdot cm^2$		
PD	ΔPD (max ΔPD)	ΔPD (10)	R	ΔR (at max PD)	ΔR (10)
Removal of Cl^- from aqueous soln. (14 expts.)					
16.2 ± 1.1	–	−13.9 ± 0.9 [a]	1.41 ± 0.12	–	0.55 ± 0.06 [a]
Removal of Cl^- from tear soln. (9 expts.)					
−0.6 ± 0.8	7.7 ± 0.7 [a]	6.3 ± 0.4 [a]	2.06 ± 0.25	0.43 ± 0.97 [n.s.]	0.80 ± 0.17 [a]

[a] $P < 0.01$; n.s. $P > 0.05$.

TABLE II

EFFECT OF CHANGING K^+ IN THE AQUEOUS SIDE IN Cl^--FREE (SO_4^{2-} SUBSTITUTED) SOLUTIONS

Values are means ± S.E. PD and R values obtained before change. Δ(max) values obtained at highest PD following change (< 10 min). Δ(10) obtained 10 min after change.

Aqueous solution		No. of Expts.	mV			$k\Omega \cdot cm^2$		
orig. (mM)	final (mM)		PD	ΔPD (max)	ΔPD (10)	R	ΔR (at max PD)	ΔR (10)
4 K^+	0 K^+	14	4.0 ± 0.8	2.2 ± 0.4 [a]	1.0 ± 0.3 [a]	2.84 ± 0.30	0.32 ± 0.08 [a]	0.37 ± 0.08 [a]
0 K^+	4 K^+	14	5.4 ± 0.9	1.1 ± 0.3 [a]	−0.8 ± 0.2 [a]	3.43 ± 0.37	0.33 ± 0.09 [a]	0.14 ± 0.05 [a]
Ouabain 10^{-4} M in aqueous solution								
0 K^+	4 K^+	6	4.8 ± 0.8	–	−1.6 ± 0.3 [a]	3.83 ± 0.70	–	−0.06 ± 0.04 [n.s.]
Amphotericin B 10^{-5} M in tear solution								
0 K^+	4 K^+	7	7.5 ± 1.7	19.9 ± 4.3 [a]	18.3 ± 4.7 [a]	2.22 ± 0.38	−0.34 ± 0.17 [n.s.]	−0.42 ± 0.15 [b]
Ouabain 10^{-4} M in aqueous solution and amphotericin B 10^{-5} M in tear solution								
0 K^+	4 K^+	7	3.8 ± 1.4	–	−2.3 ± 0.8 [b]	2.09 ± 0.53	–	−0.51 ± 0.19 [b]

[a] $P < 0.01$; [b] $P < 0.05$; n.s., $P > 0.05$.

Na^+ concentration in the cell, the anomalous PD response could be enhanced by increasing the entrance of Na^+ into the cells. This was accomplished by adding amphotericin B to the tear solution. Candia et al. [5,6] have shown that this antibiotic increases the permeability of the apical membrane to Na^+ and K^+ with the result of an increase in activity of the ($Na^+ + K^+$)-ATPase pump in Cl^--free solutions.

Effect of amphotericin B

Fig. 1 shows the typical response of PD and resistance (R) observed when the antibiotic is added to the tear solution. An initial increase in PD and decrease in R is followed by a quick return of the PD towards control with a small increase in R. The initial increase in PD and decrease in R are probably due to a marked increase in the apical Na^+ conductance [5,6].

Table III presents data on the effect of amphotericin B on PD and R in the presence of Cl^- (control solutions) and in Cl^--free (SO_4^{2-}-substituted solutions). There was a significant increase in PD which after reaching a maximum, remained elevated 10 min after addition of the drug. The 10 min increase in PD was significantly

TABLE III

EFFECT OF ADDING $1 \cdot 10^{-5}$ M AMPHOTERICIN B TO THE TEAR SOLUTION IN Cl^- AND Cl^--FREE SOLUTIONS OF FROG CORNEA

Values are means ± S.E. PD and R values obtained before change. Δ(max) values obtained at highest PD following change (< 10 min). Δ(10) obtained 10 min after change.

mV			$k\Omega \cdot cm^2$		
PD	ΔPD (max ΔPD)	ΔPD (10)	R	ΔR (at max PD)	ΔR (10)
Control soln. (20 expts.)					
17.9 ± 1.7	12.4 ± 1.5 [a]	10.4 ± 1.3 [a]	1.84 ± 0.16	−0.24 ± 0.09 [b]	−0.29 ± 0.09 [b]
Cl^--free (SO_4^{2-} subst.) soln. (7 expts.)					
5.20 ± 1.89	28.64 ± 4.72 [a]	28.59 ± 4.69 [a]	3.69 ± 0.39 [a]	−1.16 ± 0.20 [a]	−1.16 ± 0.20 [a]

[a] $P < 0.01$; [b] $P < 0.02$; n.s., $P > 0.05$.

higher in Cl^--free solutions than in Cl^- solutions (28.6 vs. 8.8 mV). The resistance decreased when amphotericin B was added to the tear solution. The decrease in resistance was significantly greater in Cl^--free solutions than in Cl^- solutions.

Anomalous PD response in the presence of Amphotericin B

As may be seen in Fig. 1, at about time 160 min the PD rapidly increased by about 20 mV when the concentration of K^+ was increased from zero to 4 mM in the absence of Cl^-. This marked, anomalous response was followed by another prompt anomalous response when K^+ was removed from the aqueous solution, the PD decreasing instead of increasing. This prompt anomalous response as a result of removal of K^+ was never observed in the presence of Cl^- and absence of amphotericin B. With $1 \cdot 10^{-4}$ M ouabain in the aqueous solution, at about 200 min in Fig. 1, no anomalous response was observed when 4 mM K^+ was added (see also Table II).

The mean increase in PD and decrease in resistance from seven experiments with amphotericin B on the tear side is shown in Table II. After a maximum increase in PD of 19.9 mV, the increase was 18.3 mV at 10 min after the addition of K^+. This increase in PD was significantly higher than in the presence of Cl^- [1] and absence of amphotericin B ($P < 0.05$). The addition of ouabain abolished the anomalous response (see Table II).

Table IV presents data on the effect of changing aqueous K^+ from 4 mM to zero in the presence of amphotericin B. In all three cases, the PD decreased significantly, which is contrary to the increase in PD observed when aqueous K^+ was decreased from 4 to 0 mM in Cl^--free solutions (Table II, top row) or in Cl^- solutions in the absence of amphotericin B [1]. In the presence of amphotericin B, the decrease in PD with removal of aqueous K^+ was significantly higher in Cl^--free than in control (Cl^-) solutions (-14.2 vs. -3.2 mV).

Data showing the inhibition by ouabain of the anomalous response in the presence of amphotericin B are presented at the bottom of Table II. In this case, adding 4 mM K^+ resulted in a normal response, that is, a significant decrease in PD. The anomalous response was completely inhibited by ouabain.

TABLE IV

EFFECT OF DECREASING STROMA K^+ FROM 4 mM TO ZERO WITH $1 \cdot 10^{-5}$ M AMPHOTERICIN B IN TEAR SOLUTION

Values are means ± S.E. PD and R values obtained before change. $\Delta(10)$ obtained 10 min after change.

mV		$k\Omega \cdot cm^2$	
PD	ΔPD (10)	R	ΔR (10)
Control Cl^- soln. (7 expts.)			
21.1 ± 3.0	−3.2 ± 1.3 [a]	0.96 ± 0.15	−0.06 ± 0.03 [n.s.]
Cl^- soln.; K^+-free tear soln. (5 expts.)			
15.3 ± 2.0	−7.7 ± 2.4 [a]	1.02 ± 0.26	−0.15 ± 0.07 [n.s.]
Cl^--free (SO_4^{2-} subst.) soln.; K^+-free tear soln. (6 expts.)			
39.1 ± 3.7	−14.2 ± 4.7 [a]	2.37 ± 0.36	0.12 ± 0.05 [a]

[a] $P < 0.05$; n.s., $P > 0.05$.

Discussion

Data presented in this paper confirm the electrogenicity of the $(Na^+ + K^+)$-ATPase pump located in the basolateral membrane of the cornea epithelium.

We have reported that in the frog cornea [1] as well as in the frog gastric mucosa [2], when the K^+ concentration is increased from 0 to 4 mM in the solution bathing the basolateral membrane (aqueous in the cornea and nutrient in the stomach), the PD increases, that is, the side at which K^+ is added becomes more positive to the other side. This anomalous PD response, contrary to that expected for a normal conductive pathway, is reproducibly obtained after the epithelium has been bathed in zero K^+ for at least 5 min for the cornea [1] and for 15 min or more for the stomach [2].

Since the anomalous PD response was completely abolished by ouabain in the case of the stomach, it was easy to explain it on the basis of an electrogenic $(Na^+ + K^+)$-ATPase pump. In the case of the cornea the anomalous response was abolished by ouabain only in about half of the experiments in Cl^- solutions. Perhaps another K^+-sensitive pathway such as an electrogenic passive KCl symport, transporting more Cl^- than K^+ ions per cycle, could also contribute to the

anomalous PD response in the cornea. As we may see in Fig. 1 and Table II, the anomalous response was also markedly decreased when the Cl^- was removed from the bathing solution. These data could support the above hypothesis of a passive electrogenic KCl symport. But, since the ($Na^+ + K^+$)-ATPase pump requires Na^+ in the cell to be activated, the cellular Na^+ may have been depleted in Cl^--free solutions, thereby rendering the pump inoperable.

That this was the case was demonstrated in the experiment with amphotericin B in the tear solution. This antibiotic is well known to increase the permeability to Na^+ of the apical membrane of epithelia [9], especially in the cornea [5,6]. Therefore, in the absence of Cl^-, Na^+ could be made available to the pump via the apical membrane by adding amphotericin B to the tear solution. As shown in Fig. 1 and Table II, the anomalous PD response was then obtained. As a matter of fact, the magnitude of the maximum increase in PD when the aqueous K^+ was increased from zero to 4 mM was significantly higher in seven amphotericin B, Cl^--free experiments (20.0 (S.E. $\pm$ 4.3) mV, Table II) than in seven Cl^- experiments ((8.8 (S.E. $\pm$ 1.2) mV) ($P < 0.05$).

To explain these results, let us analyze the circuit presented in Fig. 2, for which

$$\Delta PD = g_p/(g_p + g_{Cl} + g_K + g_X)\Delta E_p + g_K/(g_p + g_{Cl} + g_K + g_X)\Delta E_k \quad (1)$$

where g_p, g_K, g_{Cl} and g_X are the conductances ($= 1/R$) of the pump, K^+, Cl^- and X pathways, respectively (X pathway includes all but the pump, K^+ and Cl^- pathways). ΔPD, ΔE_p and ΔE_K are the changes in transepithelial PD, and changes in the pump and simple K^+ e.m.f.s, respectively, when the aqueous K^+ is increased from 0 to 4 mM (see Ref. 1 or 2 for details). For a given experimental condition, the magnitude of ΔPD will depend on the magnitude of ΔE_p and on the magnitude of its coefficient, which is the partial conductance of the pump pathway. It is our hypothesis that when Cl^- is removed from the solutions, Na^+ is depleted from the cell and ΔE_p is small or nonexistent when aqueous K^+ is increased from 0 to 4 mM. With amphotericin B, cellular Na^+ is repleted and the pump response, and therefore ΔE_p is returned to that of Cl^- conditions. Since, in the absence of Cl^-, $g_{Cl} = 0$, the partial conductance of the pump, that is, the coefficient of ΔE_p in Eqn. 2 will be greater in Cl^--free solutions than in the presence of Cl^-. Our data from amphotericin B, Cl^--free experiments are similar to those of Rang and Ritchie [10], who obtained a much greater posttetanic hyperpolarization response in SO_4^{-2} (Cl^--free) than in Cl^- media in nerve. Contrary to the gastric mucosa and cornea, at least some of the entrance of Na^+ into the nerve cytoplasm is apparently not Cl^--dependent.

Another reproducible anomalous response in the presence of amphotericin B has been the decrease in PD observed when K^+ is removed from the 4 mM K^+ aqueous solution (see Table IV). In the absence of amphotericin B the PD always increases upon the removal of K^+ from the aque-

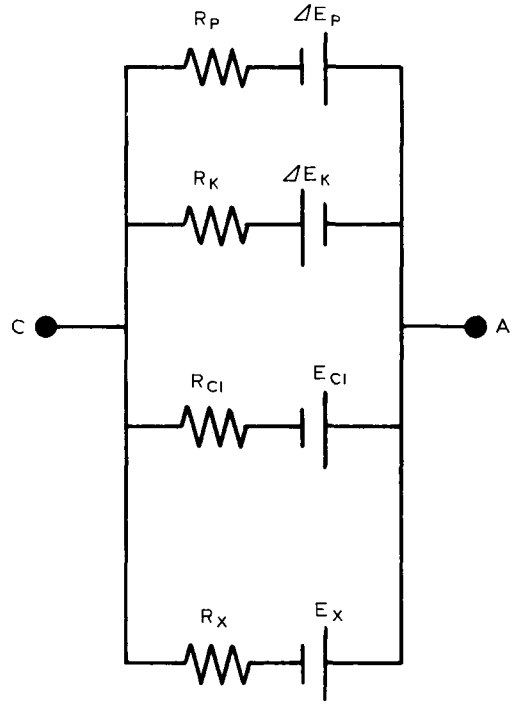

Fig. 2. Equivalent circuit for conductive pathways in the basolateral membrane of frog cornea epithelium. E_{Cl} and E_X are the e.m.f.s of the Cl^- pathway and all pathways other than Cl^-, K^+ or the pump, respectively. ΔE_p and ΔE_K are the changes in the e.m.f. of the ($Na^+ + K^+$)-pump and the simple K^+ conductive pathway when 4 mM K^+ is added to aqueous solution.

ous solution whether in the presence of Cl^- [1] or in Cl^--free solutions (see Table II). The only other experimental condition under which we have observed an anomalous response to a decrease in aqueous K^+ is in the presence of Ba^{2+} [11]. We attributed these results to the inhibition by Ba^{2+} of the simple K^+ conductance in the basolateral membrane, leaving the PD response mostly to the electrogenic $(Na^+ + K^+)$-ATPase pathway when K^+ is removed from the aqueous solution. But there is no reason to believe that amphotericin B should directly increase the resistance of the basolateral simple K^+ conductive pathway. On the other hand, amphotericin B not only increases the permeability to Na^+ across the apical membrane but also increases the apical membrane permeability to K^+ [5,6]. It is possible, that in the presence of amphotericin B, the intracellular K^+ could be decreased to the point of increasing the resistance of the simple K^+ conductance pathway in the basolateral membrane. To test this hypothesis, in order to block the exit of K^+ from the cell, the tear solution K^+ was increased to 79 mM in the presence of amphotericin B in the tear solution with Cl^- on both sides. Then, the removal of aqueous K^+ in the presence of 79 mM K^+ and amphotericin B in the tear solution resulted in a normal increase in PD. The 10 min increase in PD was 8.8 ($\pm$1.8 S.E.) mV in five experiments ($P < 0.01$). Data from these experiments support the hypothesis that the simple K^+ conductance pathway is sensitive to intracellular K^+. Of course, intracellular K^+ measurements should be done and we plan to do them in the future.

In conclusion, data presented in this paper: (1) confirm the electrogenicity of the $(Na^+ + K^+)$-ATPase pump; (2) confirm previous data that in order for Na^+ to enter the cell readily, Cl^- is needed in the aqueous solution, that is, a coupled NaCl symport may exist in the basolateral membrane of the cells; and (3) suggest there is a Na^+ concentration in the cell below which the electrogenic pump may not be operable.

Acknowledgements

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