Biochimica et Biophysica Acta, 389 (1975) 550–556

BBA 76952

DIFFUSION RESISTANCE OF ENDOTHELIUM AND STROMA OF BULLFROG CORNEA DETERMINED BY POTENTIAL RESPONSE TO K^+

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(Received September 3rd, 1974)

(Revised manuscript received January 28th, 1975)

SUMMARY

Corneas of bullfrog (*Rana catesbeiana*) were mounted between lucite chambers. A four-electrode system was used and the potential difference (PD) and the electrical resistance were measured. In intact corneas, the PD averaged 25 mV (aqueous side positive) and the electrical resistance 1.5 $k\Omega \cdot cm^2$. Perfusion of the aqueous side with high K^+ solutions resulted in a marked decrease in PD and a drop in the electrical resistance. Scraping the epithelium (leaving the stroma plus endothelium) resulted in a drop of the PD to about zero and a decrease in electrical resistance to about 0.1 $k\Omega \cdot cm^2$ and a very small PD response to a marked elevation of the K^+ concentration on the aqueous side. On the basis of the above, it is obvious that the large ΔPD in intact corneas, due to elevation of the K^+ concentration, must be due to K^+ diffusing from the aqueous side across the endothelium and stroma and reaching the epithelium. The duration of the PD response is therefore a measure of the resistance to diffusion of the stroma plus endothelium. A quantitative analysis shows that under in vitro conditions the resistance of the endothelium plus stroma to the diffusion of ions is very low.

INTRODUCTION

The primary purpose of this work is to determine, under in vitro conditions, the resistance to diffusion of ions across the endothelium and stroma of the bullfrog cornea. Previous work has shown that (a) there is a potential difference (PD) across the intact cornea with the aqueous side positive [1], (b) elevation of K^+ on the aqueous humor side results in a marked decrease in the magnitude of the PD [2] and (c) the PD across the endothelium plus stroma is essentially zero and the electrical resistance across these layers is a small fraction of the total corneal resistance. It will be shown in the present paper that marked increases in the K^+ concentration of the aqueous side produce only a small change in PD across the endothelium plus stroma. On the basis of the above it is apparent that the large transcorneal change in PD is due

to K^+ diffusing across the endothelium and stroma and reaching the epithelium where it has its effect. The time taken for the PD to reach its new level is a measure of the resistance of the endothelium and stroma to the diffusion of ions. Our method for estimating the diffusion resistance will be presented in the Discussion.

Although the primary goal of this paper is the determination of the diffusion resistance of the endothelium plus stroma, other findings are presented. For the sake of clarity we should point out that we deal with two types of resistance and they will be designated as electrical resistance and diffusion resistance.

METHODS

A four-electrode in vitro technique similar to those previously described was used [1, 3,4]. With this technique the lucite chambers are contoured to fit the curvature of the cornea so as to minimize edge damage. Except for the contouring, the chambers were symmetrical. Two electrodes are used for current sending and two for measuring the PD. The electrical resistance was determined as the change in PD after current flow of 0.5 s divided by the current [5]. The current density was 2 μA/0.36 cm^2 (0.36 cm^2 = area of cornea exposed to bathing solutions) or 5.56 $\mu A \cdot cm^{-2}$. The standard bathing solution contained in mM: Na^+, 103.5; K^+, 2.5; Cl^-, 81; HCO_3^-, 25; Ca^{2+}, 1.0; Mg^{2+}, 0.8; SO_4^{2-}, 0.8; HPO_4^{2-}, 1.0; glucose, 10. The solutions with elevated K^+ had the same composition as above except that K^+ was substituted for Na^+ (the K^+ plus Na^+ concentrations in all solutions were the same). The fluids were gassed with O_2/CO_2 (95 : 5, v/v). The PD was recorded with a recording potentiometer.

In preliminary work, we found that the technique for changing solutions of draining and refilling a given chamber was not satisfactory. This procedure itself (without changing the composition of solutions) often resulted in a change in the PD and resistance. This was true even when, to minimize bulging of the cornea, the inflow and outflow tubes of the opposite chamber were clamped during the draining and refilling.

In mounting the cornea between the in vitro chambers, one is faced with a dilemma. Too much pressure exerted by the chambers produces edge damage, too little pressure results in a poor electrical seal between the bathing solutions. In the process of draining and refilling the chamber the position of the cornea may be changed slightly, resulting in a change in the degree of the electrical sealing. The method we used for changing solutions is described in the following.

In the present experiments the fluids were not recycled; they entered the inflow tubes and after exiting via the outflow tubes were discarded. The rate of flow was 5 ml/min. In changing to a new solution the inflow on the aqueous side was switched to the new solution. Both the inflow and the outflow tubes were close to the cornea (within 1 mm) so that the cornea was exposed to the new fluid almost immediately. The advantage of this technique over the draining and refilling technique is that it is possible to maintain a constant pressure gradient across the cornea during switching from one solution to another, so as to minimize the possibility of the procedure itself changing the PD and electrical resistance. We maintained a pressure gradient of 20 cm of water (high side on the aqueous) by positioning the heights of the outflow tubes. For the control conditions we used the standard bathing solution (2.5 mM K^+)

on both the tear and aqueous sides and solution changes were made only on the aqueous side.

RESULTS

Fig. 1A shows the effects on the PD and the electrical resistance of changing the K^+ concentration on the aqueous side from 2.5 to 79 mM K^+ and return. It can be seen that there was a rapid change in both the PD (the aqueous side is normally positive) and electrical resistance and that upon return to the 2.5 mM K^+ solution the PD and resistance returned towards their control levels. Ten corneas were used and the average control PD was 25.3 mV (S.D.$\pm$7.9) and after changing to 79 mM K^+ the PD levelled off at an average value of -6.9 mV (S.D.$\pm$3.3, minus sign means aqueous side negative) and the average change in PD was 32.1 mV (S.D.$\pm$7.7). Upon return to 2.5 mM K^+ solution the average PD recovered to 19.7 mV (S.D. $\pm$6.2). The average control electrical resistance was 1.53 k$\Omega \cdot cm^2$ (S.D.$\pm$0.51) and with 79 mM K^+ was 0.85 k$\Omega \cdot cm^2$ (S.D.$\pm$0.24) the average decrease being 0.68 k$\Omega \cdot cm^2$ (S.D.$\pm$0.35). Upon returning to the 2.5 mM K^+ solution the resistance recovered to 1.50 k$\Omega \cdot cm^2$ (S.D.$\pm$0.33). The average half-time (the time for the PD to change by one-half of its total response) was for the increase to 79 mM K^+, 87 s (S.D.$\pm$32) and for the return to 2.5 mM K^+ was 164 s (S.D.$\pm$59); in every experiment the half-times were less for the elevation of the K^+ concentration than for the return response (see Discussion). All of the changes in PD, resistance and half-times were significant ("Student's" t-test with paired variates, $P < 0.01$).

Fig. 1B shows the effects of scraping the epithelium (leaving the stroma plus endothelium) on the PD and electrical resistance. It can be seen that both fell to very low values. This experiment also demonstrates that changing the K^+ concentration on the aqueous side from 2.5 to 79 mM K^+ produces only a small change in PD and

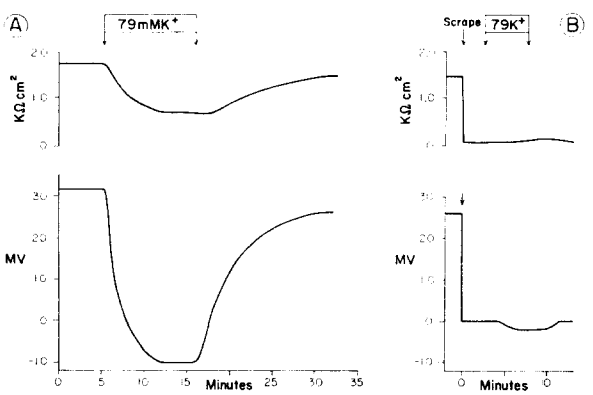

Fig. 1. (A) Effect of elevating the K^+ concentration on the aqueous side from 2.5 to 79 mM (K^+ replacing Na^+) on PD (mV) and electrical resistance (kΩ). At time of first arrow perfusion fluid changed to 79 mM K^+ and at the time of the second arrow the perfusion fluid was changed back to 2.5 mM K^+. (B) Effect of scraping epithelium, leaving stroma plus endothelium, on PD and electrical resistance. PD after scraping is about zero and the resistance is 0.1 k$\Omega \cdot cm^2$. Following scraping of the epithelium a high K^+ perfusion fluid (used during time indicated by the arrows and the box labelled 79 K^+) resulted in a decrease of the PD by 2 mV (aqueous side became more negative).

resistance across this layer. The average PD after scraping the epithelium was 0.75 mV (aqueous side positive) and the average change in PD due to increasing the K^+ on the aqueous side from 2.5 to 79 mM was −1.6 mV (aqueous side became more negative). The latter value has the orientation and is of the magnitude of the diffusion potential expected between solutions containing 2.5 and 79 mM K^+. The average electrical resistance was about 100 $\Omega \cdot cm^2$ which is not significantly different from that previously reported [1], i.e. 92 $\Omega \cdot cm^2$.

Essential to the analysis presented in Discussion is a knowledge of the relationship between the PD and the K^+ concentration of the aqueous bathing fluid. Fig. 2 shows the ΔPD vs log $[K^+]$ for a series of K^+ concentrations. It can be seen that the relationship is essentially linear with a slight tendency for the slope to be less at the lower concentrations.

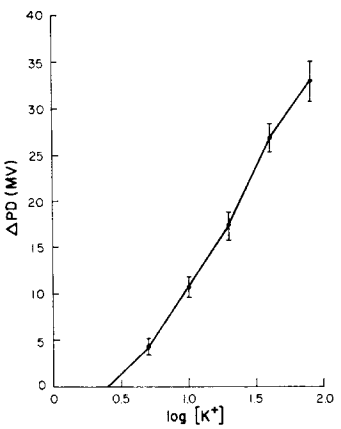

Fig. 2. Effect on PD of changing K^+ concentration of perfusion fluid from 2.5 mM to higher concentrations (5, 10, 20, 40 and 79 mM). Results are averages for five intact corneas and the vertical lines give the standard error of the mean. In all experiments following an increase in the K^+ concentration the fluid was changed back to the standard 2.5 mM K^+ solution before the next increase in the K^+. In two experiments the first increase was to 79 mM and in three experiments the first increase was to 5 mM.

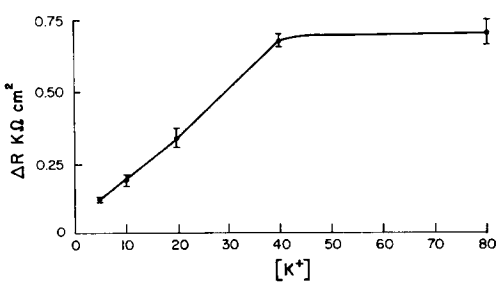

Fig. 3. Effect of elevating K^+ concentration of the aqueous side on the electrical resistance. ΔR means the original electrical resistance in 2.5 mM K^+ minus the resistance at the higher K^+ concentrations (kΩ, $[K^+]$ in mM). The electrical resistance decreases as the K^+ concentration increases from 2.5 to 40 mM and shows no further decrease with 80 mM. The values are averages for five experiments (same ones as in Fig. 2) and the vertical lines are the standard error of the means.

Fig. 3 shows the relationship between the change in electrical resistance and the K^+ concentrations of the aqueous. It can be seen that the magnitude of the decrease in resistance is approximately proportional to the K^+ concentration in the range of from 2.5 to 40 mM K^+ and that between 40 and 79 mM the response is flat. The simplest interpretation is that the resistance of a K^+ conductive mechanism, in the membrane of the epithelial cells facing the stroma, is a function of the K^+ concentration up to 40 mM, but becomes saturated at about this concentration so that further increases in the K^+ concentration do not produce further changes in the resistance.

DISCUSSION

When the K^+ concentration in the aqueous is increased (by replacing Na^+ with K^+) the main ionic movements will be the diffusion of K^+ toward the epithelium and the diffusion of Na^+ toward the aqueous solution. The force of diffusion for an ion is equal to the negative of the gradient of the electrochemical potential. However, during the period of changes in the K^+ and Na^+ concentrations, the PD across the stroma plus endothelium is essentially zero (Fig. 1B) so the force of diffusion would equal the negative of the chemical potential gradient. Therefore the K^+ concentration at the stroma-epithelial border as a function of time can be obtained to a good first approximation on the basis of a solution of Fick's diffusion equation with the proper boundary conditions.

We previously solved Fick's equation [6] for a simple diffusion barrier giving the concentration of K^+ at the end of the barrier (for the cornea the junction between the epithelium and stroma) vs time which results from a step change in the K^+ concentration at the beginning of the diffusion barrier (for the cornea, the junction between the endothelium and the fluid in the chamber on the aqueous side). The equation contains a series of exponentials (with alternating signs) but for periods equal to half-times or greater the use of a single exponential gives a good approximation and with a single exponential the equation is:

$$C = C_f - \frac{4}{\pi}(C_f - C_i)\exp\left[-\frac{\pi^2 Dt}{4a^2}\right] \tag{1}$$

where C is the concentration at the end of the barrier, t is time, C_f is the concentration of the new solution and the final concentration at the end of the barrier, C_i is the initial concentration in the barrier and that of the original bathing fluid, D the diffusion coefficient of the ion and a is the thickness of the barrier. Examination of this equation reveals that the exponential curve relating C vs t for a change from 2.5 to 79 K^+ can also represent the reverse change by a simple linear adjustment of the concentration scale. Hence if the PD were a linear function of the K^+ concentration then the exponential response resulting from a change from 2.5 to 79 K^+ should be a mirror image of the reverse change. However, the PD as shown in Fig. 2 is a function of the logarithm of the K^+ concentration and hence the responses are not mirror images. For example, it takes essentially the same time for the concentration to increase from 2.5 to 12.5 mM K^+ (a ΔK^+ of 10 mM) as for the concentration to decrease from 79 to 69 mM K^+ and obviously log 12.5/2.5 > log 79/69. It would therefore be predicted that, in going from low to high concentration, the response of

the PD would be more rapid than in going in the reverse direction, which is what was found experimentally.

We can define the resistance to diffusion by analogy to Ohms Law as equal to the ratio of the force of diffusion to the rate of diffusion. However, we can also estimate the resistance to diffusion by the following method in which calculations are made for the thickness of a homogeneous diffusion barrier which has the same half-times as those found experimentally for the stroma plus endothelium.

From Fig. 2 we see that the PD is to a good approximation a linear function of the log of the K^+ concentration, hence:

$$V = G \ln \frac{C_c}{C} \tag{2}$$

where V is the PD, C_c is the epithelial cellular K^+ concentration, C is the K^+ concentration outside the cells at the stromal-epithelial junction and G is defined by this equation. For a change in PD, $\Delta V = V_i - V$ (where V_i is the initial PD before the change in K^+ concentration) we have

$$\Delta V = G \ln \frac{C}{C_c} - G \ln \frac{C_c}{C} \tag{3}$$

hence

$$\Delta V = G \ln \frac{C_i}{C} \tag{4}$$

When $C = C_f$ in Eqn 4 the ΔPD is designated by ΔV_f. When the PD changes by half its total change $\Delta V = \Delta V_{0.5}$ and $C = C_{0.5}$. Since ΔV_f, C_f and C_i are known for both increases and decreases in the K^+ concentration, the G in Eqn 4 is easily calculated for the changes in K^+. For the increase in K^+ ($C_i = 2.5$ and $C_f = 79$ mM K^+) $G = 9.32$ while for the decrease in K^+ ($C_i = 79$ and $C_f = 2.5$ mM K^+) $G = -7.7$ (note that the natural log (ln) is used). From the half-time values (87 s for the increase and 164 s for the decrease, vide supra) of the average ΔV, the concentrations of K^+ at the stromal-epithelial border can be calculated from Eqn 4 and for the increase of K^+, $C_{0.5} = 14.1$ mM and for the return $C_{0.5} = 14.0$ mM. All of the values in Eqn 1 are now known except D/a^2 and this ratio was calculated and was found to be for the increase in K^+, $1.9 \cdot 10^{-3}\ s^{-1}$ and for the decrease in K^+, $5.3 \cdot 10^{-3}\ s^{-1}$. Using the value of D for K^+ from previous work on the connective tissue of the gastric mucosa [6] ($D = 0.5 \cdot 10^{-5}$ cm^2/s) which is about one-third the value found for free solution we find that the calculated thickness a is 512 and 307 μm for the increase and decrease in K^+, respectively. The average value is 410 μm.

For the purposes of our analysis we can use either of the above values or the average value. However, the rate of flow of fluid through the aqueous chamber was 5 ml/min and the change in the K^+ in the aqueous chamber is not instantaneous so that the above values for a would be overestimated and more so for the change from 2.5 to 79 mM K^+ than for the reverse change so that the value 307 μm is more reliable than the value of 512 μm. However, as pointed out above either of the values or their average can be used for our purposes. Various values have been given for the actual (histological) thickness of the bullfrog cornea ranging from 110 [7] to 371 μm [8]; in our hands the actual thickness of the stroma plus endothelium is about 150 μm.

However, there is of course an unstirred layer of appreciable thickness on the aqueous side of the endothelium so that the average thickness of the stroma and endothelium plus unstirred layer is of the order of magnitude of the calculated thickness for the stroma plus endothelium. In other words, the calculations indicate that the endothelium plus stroma behave like the connective tissue of the gastric mucosa where there is no continuous cell layer. Therefore, we conclude that the endothelium does not significantly retard the diffusion of ions from the aqueous to the epithelium.

Previously it was found that histological studies on corneas, used in in vitro experiments, revealed no obvious damage to the endothelium [1]. However, if 1 % (or less) of the endothelium were damaged and the cells replaced with a fluid of the same electrical conductivity as the bathing fluid, it is easily shown that the electrical resistance and the resistance to the diffusion of ions across it would be vanishingly small so even though we exercised great care not to damage the cornea we cannot eliminate the possibility that under our in vitro conditions the low resistance of the endothelium to the diffusion of ions may be due to injury.

If in our experiments the endothelium were not damaged then if we assume that in the frog cornea the endothelium plays a role in the maintenance of stromal deturgesence, as had been shown for the mammalian cornea [9], we must search for possible mechanisms that would be applicable to a very leaky membrane. Before considering possible mechanisms for a leaky membrane it is obviously important to make sure the endothelium possesses these characteristics. This could be accomplished by studies on an intact globe preparation where the possibility of damage to the endo thelium is essentially eliminated and our orientation is that work on the intact glob- takes priority over continued work along the present lines with the in vitro techniquee

ACKNOWLEDGEMENT

Supported by NIH 5R01 EY00456 and NSF GBMS-72-02508.

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