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QUANTITATION OF CORNEAL ENDOTHELIAL POTENTIALS USING A CARBOCYANINE DYE

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The carbocyanine dye, diS-C_3-(5) was used to quantitate the plasma membrane potential of the bullfrog corneal endothelium. It was shown that valinomycin hyperpolarized the endothelial cell and that in the presence of the ionophore the membrane potential largely reflected the K^+ equilibrium potential. Using calibration curves constructed by changing medium K^+ concentration in the presence of valinomycin, and nigericin and ouabain to abolish ion gradients and electrogenic pump activity, the cell membrane potential was calculated to be 28.6±4.2 mV. The major source of this potential was a K^+ diffusion potential, and the membrane Na^+ conductance reduced the cell potential to less than the apparent K^+ equilibrium potential of 51.5±5.1 mV. About 20% of the cell potential could be ascribed to the rheogenic ($Na^+ + K^+$)-ATPase.

Introduction

The electrical characteristics of the corneal endothelium have been difficult to define by standard techniques. This is due to the leakiness of the cellular junctions and the small size of the cells. This latter property has impeded progress with microelectrode exploration of the membrane properties of the endothelium cell. Recently, however, some publications [1,2] have appeared which define the mean cell potential as about 30 and 40 mV, respectively, in the rabbit corneal endothelium.

The transparency of the tissue has allowed a completely different approach to the question. Carbocyanine dyes, shown to be useful probes of potential gradients across membranes of various cells [3,4] and organelles, also proved suitable for measurement of certain of the electrical characteristics of the endothelial cell layer. For example, it was shown that the dye responded to changes in electrical potential of the endothelium when added to the aqueous side of the cornea and that this was so especially for the cornea stripped of epithelium. The previous publication [5] was largely a qualitative demonstration of the method proving the assessibility of the electrical properties of this tissue to dye probe methods. In this report we attempt to quantitate cell potentials and determine their origin. The small volume of the endothelium precludes any meaningful measurement of ionic content by standard methods of flame photometry and discrimination between cellular and extracellular compartments. Accordingly we have used a combination of ionophores such as valinomycin and nigericin and the ($Na^+ + K^+$)-ATPase inhibitor ouabain to determine the magnitude and basis of endothelial cell potentials.

Abbreviation: diS-C_3-(5), 3,3′-dipropylthiodicarbocyanine iodide.

Materials and Methods

The carbocyanine dye diS-C_3-(5) was obtained from Dr. Alan Waggoner. The neutral exchange ionophore, nigericin, was obtained from Eli Lilly

and ouabain and valinomycin from Sigma. All other chemicals were of the highest purity available.

Bullfrog corneas were used as previously described [5]. In summary, the frog was decapitated and the epithelium removed from the cornea by rubbing with coarse paper. The cornea was removed and mounted in an in vitro chamber so designed that it could be placed in the sample compartment of a Perkin-Elmer MPF-44 fluorimeter with the cornea at an angle of 45° to the incident light. The aqueous side was perfused by means of a peristaltic pump so that solution composition could be changed.

The dye was perfused through the chamber at a concentration of 4 μM for 10 min, in frog Ringer following which, perfusion with frog Ringer with no dye was started. The solution contained in mM: K^+ 2.5, Na^+ 103.5, Cl^- 81, HCO_3^- 25, Ca^{2+} 1.0, SO_4^{2-} 0.8, PO_4^{3-} 1.0 and glucose 10. Ionic changes were made by increasing K^+ at the expense of Na^+, changing Na^+ for choline and SO_4^{2-} for Cl^-. Ouabain was added at a final concentration of 10^{-3} M, valinomycin at 10^{-5} M and nigericin at 20 μg/ml. The excitation and emission wavelengths were 622 and 689 nm, respectively, and the fluorescence was recorded on a strip chart recorder. The experiments were performed at 22°C.

The overall experimental design was to carry out ion concentration changes in the absence and presence of the K^+ selective ionophore valinomycin to establish the K^+ electrode characteristics of the treated and untreated tissue. Nigericin was used to abolish the Na^+ and K^+ gradients of the endothelium and ouabain was used to determine the electrogenicity of the $(Na^+ + K^+)$-ATPase in this particular tissue.

Results

1. Effect of valinomycin

When the K^+ concentration was changed on the aqueous side of the endothelium there was an increase in fluorescence corresponding to a depolarization of the cell layer. Fig. 1 shows the response of an experiment in Table I due to a change of $[K^+]$ from 2.5 to 79 mM K^+ (K^+ replaced Na^+), namely a 31.6-fold change in concentration at constant Cl^-. Upon changing to regular $[K^+]$, the fluorescence change was reversed. When valinomycin was added at 10^{-5} M there was a decrease in fluorescence corresponding to a hyperpolarization of the cell membrane. Higher concentrations of valinomycin had no further effect. That the relative K^+ conductance was enhanced by the ionophore is shown by the smaller fluorescence enhancement induced by the same K^+ change preceding valinomycin treatment. The average change of eight experiments was 33.9 units before and 46.1 units after valinomycin treatment. Thus a K^+ diffusion potential contributes significantly to the cell membrane potential, and valinomycin increases the K^+ conductance of the membrane relative to the other ionic conductances which reduce the potential below that of the K^+ equilibrium potential in the absence of valinomycin.

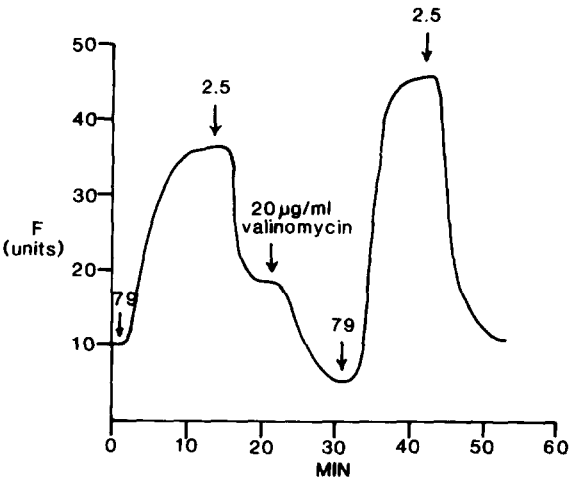

Fig. 1. A large increase in fluorescence (depolarization) was observed on changing the aqueous medium K^+ from 2.5 mM to 79 mM. On returning the medium to 2.5 mM there was a decrease in fluorescence (repolarization). Subsequent addition of valinomycin produced a further decrease in fluorescence (hyperpolarization). Repeating the pulsing of K^+ in the presence of valinomycin produced a larger increase in fluorescence than in its absence.

2. Effect of Cl^- changes

When the Cl^- concentration of the medium was changed from 81 to 10 mM with SO_4^{2-} substitution, no change in fluorescence was found

TABLE I

THE EFFECT OF VARIOUS K^+ CONCENTRATIONS IN THE AQUEOUS PERFUSING MEDIUM ON FLUORESCENCE AFTER VALINOMYCIN

Nigericin and ouabain were used to dissipate the potential. Means and S.E. of five experiments are given.

	ΔF (units)						
	Valinomycin	K^+					Nigericin + ouabain
		10 mM	25 mM	50 mM	75 mM	75 – 25	
Mean	15.2	6.2	19.6	33.4	41.0	20.3	36.5
S.E.	2.236	0.490	2.891	2.713	3.674	0.750	0.645

either before or after valinomycin treatment. Hence Cl^- conductance makes an insignificant contribution to the membrane potential of the endothelial cell either in the presence or absence of valinomycin.

3. Effect of Na^+ changes

To determine the effect of valinomycin on the Na^+ conductance, with 0 mM K^+ on the tear side, the Na^+ concentration of the aqueous bathing fluid was changed from 103.5 to 27 mM (choline replaced Na^+). The average change in fluorescence of three experiments before valinomycin was 6.2 units of hyperpolarization (decreased fluorescence). After the addition of valinomycin the average change was 2.8 units. No changes were observed when the stroma alone was mounted in the chamber. Since the residual changes occur in the presence of ouabain it cannot be ascribed to changes of pump activity. Hence valinomycin reduced but did not abolish the dye response to changes of Na^+ concentration in the aqueous medium.

4. Calibration of fluorescence

The technique was to carry out step changes in K^+ concentration followed by return to control conditions in the presence of valinomycin. Fig. 2 shows a typical experiment. Firstly, the characteristic hyperpolarization due to valinomycin is shown. Then with successive K^+ changes there is a progressive increase in the fluorescence as a function of increasing changes in K^+ concentration. When the average of five experiments (Table I) is plotted in terms of the fluorescence change as a function of the log of medium K^+ concentration, Fig. 3, it can be seen that the plot is linear between 25 and 75 mM K^+ with a fall off below 25 mM K^+. This non-linearity at low concentration of K^+ is often found in such experiments in other cell types [6].

Using the linear range of the curve in Fig. 3, the conversion of arbitrary fluorescence units to potential was straightforward. Thus for a change of medium K^+ between 25 and 75 mM, the fluorescence change, ΔF, corresponds, applying the Nernst equation, to a change in potential of 28.6 mV. This allows direct conversion of change in fluorescence to change in potential difference. The error due to the residual Na^+ conductance, given a 2.6-fold change in Na^+ concentration with the K^+ changes made, was less than 10%.

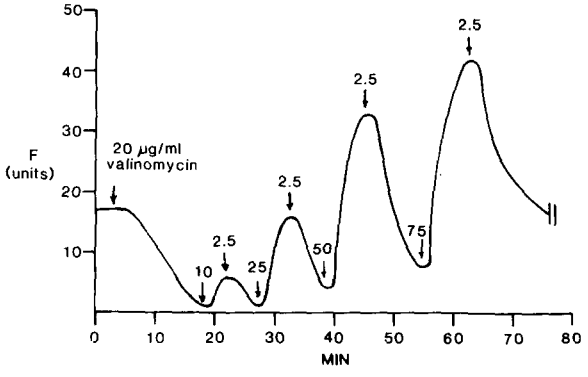

Fig. 2. A potassium electrode effect (hyperpolarization) was produced when valinomycin is added to the aqueous medium. The aqueous medium was then changed by pulsing with stepwise increases of K^+ (10 mM K^+, 25 mM K^+, 50 mM K^+ and 75 mM K^+) which produced a larger increase in fluorescence with each change.

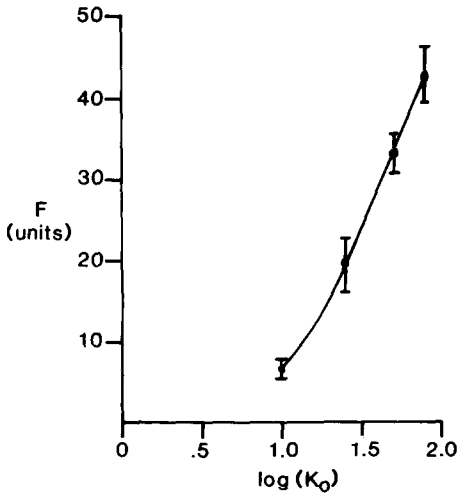

Fig. 3. A log plot of the concentration changes of the aqueous medium K^+ versus the fluorescence changes from Fig. 2 is shown. The slope of the line was linear from 25 mM to 75 mM but is not linear below 25 mM.

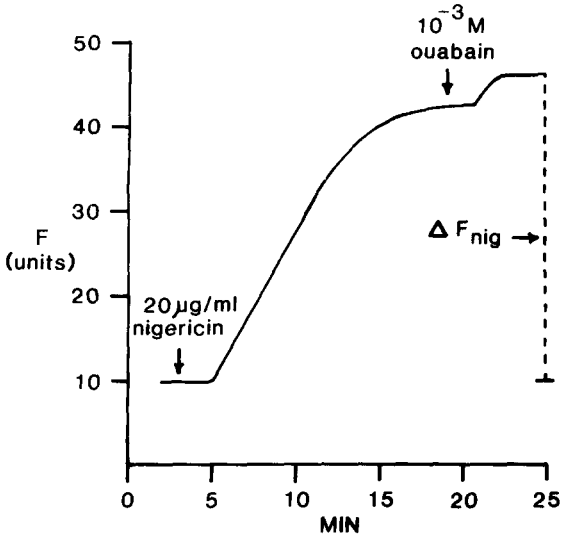

Fig. 4. Nigericin, a neutral exchange ionophore for Na^+ and K^+, is shown to produce a larger increase in fluorescence as the ionic gradients of the membrane are dissipated. After reaching a nadir, the metabolic inhibitor ouabain is added to inhibit the $(Na^+ + K^+)$-ATPase. A further increase in fluorescence is observed and the cumulative total increased fluorescence corresponds to zero membrane potential.

5. Measurement of cell potential

If the cell concentration of K^+ was measurable, then conversion of the fluorescence to millivolts potential of the tissue in the presence of valinomycin would be directly possible. Since it is not, the change in fluorescence due first to the addition of nigericin was measured. This change in fluorescence is due to the ionophore-induced dissipation of the Na^+ and K^+ gradients across the cell membrane. A typical result is shown in Fig. 4. There was a large increase in fluorescence due to nigericin as expected. If ouabain was added to inhibit $(Na^+ + K^+)$-ATPase and eliminate any contribution the rheogenic properties of this pump might have to the cell potential, a second much smaller increase in fluorescence was found. Since both K^+ and Na^+ conductances are present, a Donnan distribution would not discriminate between these ions, and nigericin would abolish Na^+ and K^+ gradients. The lack of effect of valinomycin under these conditions supports this conclusion. The total change in fluorescence due to nigericin and ouabain can now be converted to the value of the cell potential from the calibration curve using the calibrated fluorescence signal. The cell potential by this means was 28.6 ± 4.2 mV ($n = 4$). The contribution of the ionic diffusion potentials was 24.0 mV and that of the $(Na^+ + K^+)$-ATPase was 4.6 mV.

Discussion

The use of potential sensitive dyes has provided interesting and quantitative data with cells such as erythrocytes [7] and ascites tumor cells [8] and in organelles such as gastric vesicles [9], to cite just a few examples. To our knowledge the cornea was the first epithelial tissue where the use of carbocyanine dyes had been shown to be useful, even essential for measurement of cell potentials [5]. However, as with ascites cells or vesicles, quantitation of the fluorescence response expands the usefulness of this technique with the cornea. In most circumstances when these dyes have been used, valinomycin is used to produce a Nernst potential response to medium K^+ changes. Either the K^+ concentration is changed subsequent to the addition of valinomycin as done here, or valinomycin is added to cells suspended in a series of media at different K^+ concentrations. Knowing the cellular K^+ concentration, the concentration in the

medium at which valinomycin produces no change in fluorescence, the null point, allows direct calibration of the K^+ equilibrium potential which, at the null point, is the same as the cell membrane potential in the absence of valinomycin. Such a method would require multiple experiments on the cornea, as well as knowledge of cell [K^+], hence the approach of changes of K^+ concentration was employed. In both instances it is important to show that valinomycin has indeed brought the cell membrane potential close to the K^+ equilibrium potential. This appears to be the case in the cornea since no evidence was obtained for Cl^--dependent changes in fluorescence in the absence of medium K^+ and only a small change was obtained for Na^+ in the presence of valinomycin. The response of the fluorescence to changes in medium was log [K^+] linear over a considerable range of K^+ concentrations, hence calibration of the dye signal was possible.

The initial uptake of the dye could not be measured to allow estimate of cell potential since the tissue was preloaded with dye before the experimental changes. Further we have shown that the stroma does take up some dye but this uptake is not sensitive to ion changes in the bathing medium. Hence a measurement of uptake of added dye would probably be less accurate than a method designed to eliminate plasma membrane potential and measure the subsequent change in fluorescence.

Nigericin has been shown to abolish gradients of K^+ and Na^+ in the presence of ouabain in, for example, isolated rabbit gastric glands [10] and ascites cells [11]. Presumably its action is equivalent in the endothelial cell. Since there was no change in fluorescence when valinomycin was added subsequent to ouabain and nigericin, it would appear that K^+ is at equilibrium after this treatment of the tissue. This experimental maneuver then allows calculation of the endothelial cell potential and its origin. Thus a major contribution to the 28 mV potential is made by the K^+ diffusion potential, but this is partially shunted by an Na^+ conductance. Comparing the change in fluorescence for a 4-fold change in Na^+ to that for an equivalent change for K^+, namely 6 fluorescence units as compared to 20 fluorescence units, the P_{Na^+}/P_{K^+} ratio can be estimated to be about 0.3. This ratio falls to 0.1 in the presence of valinomycin. The effect of ouabain, in inducing a 5 mV depolarisation, shows that a 20% contribution is made by the ($Na^+ + K^+$)-ATPase to the cell membrane potential.

Acknowledgements

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