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The Electrogenic Sodium Pump of the Frog Retinal Pigment Epithelium

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Summary. It was previously shown that ouabain decreases the potential difference across an *in vitro* preparation of bullfrog retinal pigment epithelium (RPE) when applied to the apical, but not the basal, membrane and that the net basal-to-apical Na^+ transport is also inhibited by apical ouabain. This suggested the presence of a Na^+-K^+ pump on the apical membrane of the RPE. In the present experiments, intracellular recordings from RPE cells show that this pump is electrogenic and contributes approximately -10 mV to the apical membrane potential (V_{AP}). Apical ouabain depolarized V_{AP} in two phases. The initial, fast phase was due to the removal of the direct, electrogenic component. In the first one minute of the response to ouabain, V_{AP} depolarized at an average rate of 4.4 ± 0.42 mV/min ($n=10$, mean $\pm$SEM), and V_{AP} depolarized an average of 9.6 ± 0.5 mV during the entire fast phase. A slow phase of membrane depolarization, due to ionic gradients running down across both membranes, continued for hours at a much slower rate, 0.4 mV/min. Using a simple diffusion model and K^+-specific microelectrodes, it was possible to infer that the onset of the ouabain-induced depolarization coincided with the arrival of ouabain molecules at the apical membrane. This result must occur if ouabain affects an electrogenic pump. Other metabolic inhibitors, such as DNP and cold, also produced a fast depolarization of the apical membrane. For a decrease in temperature of $\simeq 10\,°C$, the average depolarization of the apical membrane was 7.1 ± 3.4 mV ($n=5$) and the average decrease in transepithelial potential was 3.9 ± 0.3 mV ($n=10$). These changes in potential were much larger than could be explained by the effect of temperature on an RT/F electrodiffusion factor. Cooling the tissue inhibited the same mechanism as ouabain, since prior exposure to ouabain greatly reduced the magnitude of the cold effect. Bathing the tissue in 0 mM $[K^+]$ solution for 2 hr inhibited the electrogenic pump, and subsequent re-introduction of 2 mM $[K^+]$ solution produced a rapid membrane hyperpolarization. We conclude that the electrogenic nature of this pump is important to retinal function, since its contribution to the apical membrane potential is likely to affect the transport of ions, metabolites, and fluid across the RPE.

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The retinal pigment epithelium (RPE) transports ions and metabolites between the retina and its choroidal blood supply (Noell, 1952; Dowling, 1960; Noell, Crapper & Paganelli, 1964; Lasansky & DeFisch, 1966; Young, 1969; Steinberg & Miller, 1973; Miller & Steinberg, 1976, 1977*b*). This transport is a consequence of the functional and structural asymmetries of the RPE cell membranes that face the retina and the choroid; the *apical* and *basal* membranes, respectively (Miller & Steinberg, 1977*a*). An important example of this asymmetry is the sensitivity of the apical membrane to ouabain. In an *in vitro* preparation of the RPE, ouabain alters the membrane potentials when it is applied to the apical membrane but has no effect when applied to the basal membrane (Steinberg &Miller, 1973).

The sensitivity of the apical membrane to ouabain strongly suggests that there is a ($Na^+ - K^+$)-ATPase, i.e., a $Na^+ - K^+$ pump, on the apical membrane (Skou, 1965; Quinton, Wright & Tormey, 1973; Schwartz, Lindenmayer & Allen, 1975; Ernst & Mills, 1977). This is further supported by the finding of an active transport of sodium across the RPE in the choroid-to-retina direction (Miller & Steinberg, 1977*b*) and of potassium and rubidium in the opposite direction (Miller & Steinberg, *unpublished*). Both of these active movements are inhibited by apical, but not basal, ouabain. The present paper further characterizes the $Na^+ - K^+$ pump of the apical membrane by showing that it is electrogenic, hyperpolarizing the resting potential of the apical membrane by an average of 9.6 mV. This pump is important, because it helps to maintain both the intracellular composition of the RPE cells and the extracellular composition of the space between the RPE apical membrane and the photoreceptors.

Materials and Methods

These studies were carried out on the isolated retinal pigment epithelium-choroid of the bullfrog, *Rana catesbeiana*. The RPE consists of a single layer of cuboidal epithelial cells. The basal surface of the RPE faces the choroid, which consists mainly of blood vessels and melanocytes dispersed in a fibrous stroma. Individual RPE cells measure approximately 15 μm in width and depth (Porter & Yamada, 1960; Nilsson, 1964; Steinberg, 1973). The junctional complexes connecting these cells in frogs differ from those of other species by being absent at the cellular apices; they begin about halfway down the lateral surfaces of each cell (Porter & Yamada, 1960; Hudspeth & Yee, 1973). The apical surface of the RPE faces the sensory retina, and is covered with villous-like processes that are 60–95 μm long. These processes are closely apposed to the photoreceptors, and extend all the way down to their inner segments (Nilsson, 1964).

In these *in vitro* studies, the tissue was mounted as a membrane separating two separate fluid compartments, which were continuously perfused with a modified Ringer's solution. The fluid bathing the apical surface was referred to as the apical solution, while that bathing the basal surface was referred to as the basal solution. The composition of these two solutions could be separately controlled.

Electrophysiology

The chamber design and the techniques used for dissecting the tissue and mounting it between two Lucite plates were identical to those used in a previous study (Miller & Steinberg, 1977*a*). In both the apical and basal chambers, a calomel electrode made electrical contact with the perfusion solution via a Ringer-agar bridge. The transepithelial potential, TEP, was recorded (apical side positive) differentially between the two calomel electrodes. Membrane potentials from RPE cells were recorded with micropipette electrodes, with respect to either the apical or basal calomel electrode. When referred to the apical calomel electrode, the voltage measured was the apical membrane potential, V_{AP}, and when referred to the basal calomel electrode, the voltage measured was the basal membrane potential, V_{BA}. The two potentials, V_{AP} and V_{BA}, could be recorded as one signal by alternating between the two calomel references at 20 Hz (Miller & Steinberg, 1977*a*). Since the pigment epithelial membrane potentials change relatively slowly, no significant information was lost in this multiplexing process. In some figures (e.g., Fig. 2), the combined (V_{AP}, V_{BA}) signal is displayed on a slow time base, so that V_{BA} and V_{AP} form the upper and lower envelopes, respectively, of the waveform. The construction and use of the double-barrel, K^+-specific microelectrodes were previously described in detail (Oakley, 1977). In some experiments the temperature of the tissue was monitored with a thermistor (Yellow Springs Instruments) in the basal solution. The illustrations are either photographic copies of penwriter (Brush 220) recordings, or computer-drawn plots of FM tape recordings (Oakley, 1977).

Solutions

The steady-state perfusing solution was a modified Ringer's solution having the following composition (in mM): 82.5 NaCl, 2.0 KCl, 27.5 $NaHCO_3$, 1.0 $MgCl_2 \cdot 6H_2O$, 1.8 $CaCl_2$, and 10.0 glucose. The osmolarity of this solution was 227 mosM. In some experiments, the soldium concentration was varied by equimolar replacement of NaCl and $NaHCO_3$ with Tris chloride [Tris (hydroxymethyl) aminomethane hydrochloride] and choline bicarbonate, respectively. In other experiments, the potassium concentration was varied by equimolar exchange of KCl and NaCl. All solutions were continuously gassed with 95% O_2/5% CO_2, and their pH was 7.4 ± 0.1.

Results

Electrical Components of the Model

The passive electrical properties of the apical and basal membranes of the isolated frog RPE were previously analyzed using an equivalent circuit similar to the one shown in Fig. 1 (Miller & Steinberg, 1977*a*). In

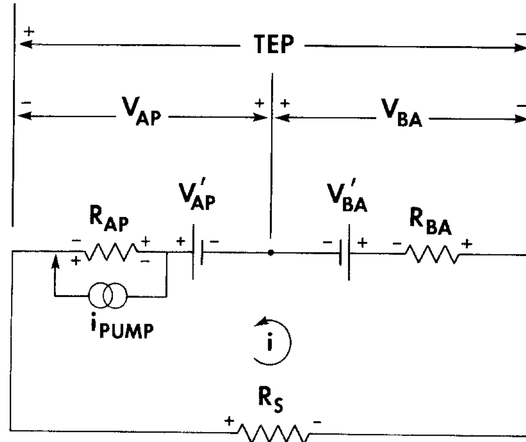

Fig. 1. Equivalent circuit for the retinal pigment epithelium. The apical membrane is represented by a resistor (R_{AP}) in series with a battery (V'_{AP}). Similarly, the basal membrane is represented by a resistor (R_{BA}) in series with a battery (V'_{BA}). The membrane resistances are shunted by a resistor (R_S) as described in the text. Due to the difference between V'_{AP} and V'_{BA}, a steady current (i) flows through the circuit in the counter-clockwise direction. The electrogenic pump on the apical membrane is modelled as a current source which produces a constant current (i_{pump}). As a result of current flow across the apical and basal membrane resistances, the potentials recorded across them (V_{AP} and V_{BA}, respectively) differ from the batteries (V'_{AP} and V'_{BA}, respectively). The steady potential across the tissue is called the transepithelial potential (TEP). The potentials V_{AP}, V_{BA}, and TEP are labeled with the polarity with which they were recorded. In all figures, these potentials are displayed with positive polarity upwards

this circuit, the apical membrane is modelled as a resistance, R_{AP}, in series with a battery, V'_{AP}. Similarly, the basal membrane is modelled as a resistance, R_{BA}, in series with a battery, V'_{BA}. The pigment epithelial membrane resistances are shunted by a resistor, R_S, which represents the parallel combination of the resistance of damaged cells at the edge of the tissue and the paracellular resistance across the intercellular junctional complexes. When both membranes are perfused with the standard Ringer's solution, a steady current, i, flows through the circuit in the counter-clockwise direction, since V'_{AP} is greater in magnitude than V'_{BA} (Miller & Steinberg, 1977*a*). This current hyperpolarizes the basal membrane and depolarizes the apical membrane. Thus, the potentials recorded by an intracellular microelectrode, V_{AP} and V_{BA}, differ in absolute magnitude from the membrane batteries, V'_{AP} and V'_{BA}, respectively. The transepithelial potential (TEP) is equal to $V_{BA} - V_{AP}$, and the transepithelial resistance, R_T, is equal to $\frac{R_S(R_{AP}+R_{BA})}{R_{AP}+R_{BA}+R_S}$.

It will be shown that the apical membrane also contains a separate source of steady current, i_{pump}, which results from a hyperpolarizing, electrogenic pump. Part of the pump current flows through R_{AP}, hyperpolarizing the apical membrane, and part of it flows through R_S and then through R_{BA}, hyperpolarizing the basal membrane. The major fraction of the pump current flows across the apical membrane, since R_{AP} is much smaller than the sum of R_S and R_{BA} (Miller & Steinberg, 1977*a*). The pump current hyperpolarizes the apical and basal membranes by

$$\Delta V_{AP} = i_{pump} \left[\frac{R_{AP}(R_{BA}+R_S)}{R_{AP}+R_{BA}+R_S} \right] \tag{1}$$

and

$$\Delta V_{BA} = i_{pump} \left[\frac{R_{BA} \cdot R_{AP}}{R_{AP}+R_{BA}+R_S} \right]. \tag{2}$$

Ouabain-Induced Potential Changes

If the apical membrane contains a hyperpolarizing, electrogenic pump, then inhibiting the pump should cause membrane depolarizations with two distinct phases, one much faster than the other (Gorman & Marmor, 1974). The fast phase will represent the termination of the pump's direct contribution to the membrane potential; its time of onset will be limited by the time it takes the ouabain molecules to reach the apical membrane and bind to the pump sites. Since, in squid axon, ouabain molecules are more than 50% bound in less than 7 sec (Baker & Manil, 1968; Baker & Willis, 1972), it is assumed that the time course of this fast phase will be determined mainly by the time it takes ouabain molecules to reach the apical membrane. The second, slower phase will occur as the sodium and potassium ionic gradients run down across both the apical and basal membranes. Experimentally, the distinction between these two phases could be obscured by such factors as the magnitude of the electrogenic response (small, if the membrane resistance is small) and the time constants for passive equilibration of the ionic gradients (rapid, if the permeabilities and surface/volume ratios are large).

The effects of adding ouabain, 10^{-4} M, to the apical solution are shown in Fig. 2. Membrane potential changes appear in the lower part of Fig. 2; the bottom edge of the trace is the apical membrane potential (V_{AP}), and the top edge is the basal membrane potential (V_{BA}). The difference between the two membrane potentials is the transepithelial

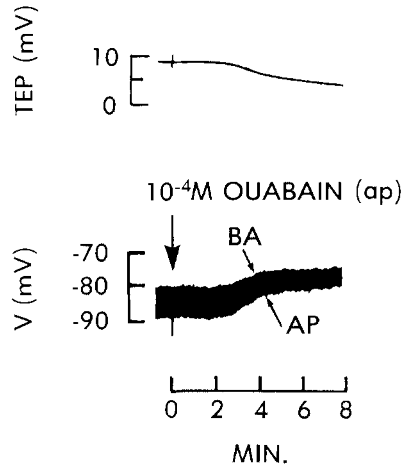

Fig. 2. Changes in V_{AP}, V_{BA}, and TEP as a result of perfusion of the apical membrane with 10^{-4} M ouabain. In this and in several subsequent figures, the lower envelope of the intracellular voltage trace is V_{AP} and the upper envelope is V_{BA}. The difference between V_{BA} and V_{AP} is equal to the TEP. At $t=0$, the solution perfusing the apical membrane was switched to a Ringer's solution containing 10^{-4} M ouabain. These responses were superimposed upon a V_{AP} of -89 mV and a TEP of $+9$ mV

potential (TEP), which appears alone in the upper part of the figure. A fast depolarization of V_{AP} began $\simeq 2$ min after the addition of ouabain, reaching a level of 8–9 mV in 2 min. In ten tissues, V_{AP} depolarized at an average rate of 4.4 ± 0.42 mV/min (mean $\pm$ SEM) during the initial one minute of the response to ouabain, and depolarized an average of 9.6 ± 0.5 mV (mean $\pm$ SEM) during the entire fast phase. Following this *fast* phase, the apical membrane potential continued to depolarize for hours at a much slower rate; the average rate of depolarization during this *slow* phase was 0.4 mV/min.

As indicated by Eq. (2), the depolarization of the basal membrane, observed during the fast phase of the ouabain response, resulted from a decrease in pump current flowing across R_{BA}. If R_S, the shunt resistance, is made sufficiently large, and/or R_{BA}, the basal membrane resistance, is made sufficiently small, then the fast phase of the basal membrane depolarization should become negligibly small. This prediction was tested as follows.

It was possible to increase R_S, and thus decrease the electrical coupling of the apical and basal membranes, by perfusing the tissue with a low $[Na^+]$ Ringer's solution. In order to demonstrate the effectiveness of this uncoupling, changes in membrane potential were recorded in

response to an increase in potassium ion concentration in the apical solution, both in normal and low [Na^+] solution. It has been shown previously that the presence of the shunt resistance (R_S) causes K^+-induced changes in potential that are generated at one membrane to produce changes in potential across the opposite membrane (Miller & Steinberg, 1977*a*).

A tissue was first perfused with the standard Ringer's solution, and, as expected, both the apical and basal membranes depolarized in response to an increase in apical potassium ion concentration from 2 to 55 mM, as shown in the upper part of Fig. 3. The tissue was then perfused on both sides with 11 mM [Na^+] Ringer's solution (*see Materials and Methods*). After 1 hr, the trans-epithelial resistance, R_T, had increased from 4,500 to 10,000 Ω. While keeping the sodium concentration equal to 11 mM, the potassium concentration in the apical solution was again increased from 2 to 55 mM, as shown in the lower part of Fig. 3. In contrast to the previous result, there was no change in potential across the basal membrane. Both this result and the increase in R_T were most likely due to an increase in R_S, which electrically uncoupled the apical and basal membranes.[1]

The responses in Fig. 4, recorded from the same cell as in Fig. 3, show that in 11 mM [Na^+] Ringer's solution, the addition of ouabain to the apical solution only affected the apical membrane potential. Even after 10 min perfusion with ouabain, the basal membrane had not depolarized. This result would be expected if the fast phase of depolarization originated solely at the apical membrane and there was little or no shunting of the depolarization to the basal membrane. In contrast, *both* membranes should have depolarized if the fast phase was caused by ionic gradients running down. In fact, 15 min after the addition of ouabain, both the apical and basal membranes began to depolarize slowly ($\simeq$0.45 mV/min), indicating that the ionic gradients were running down across both membranes.

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1 The low sodium treatment also increased the voltage divider ratio, R_{AP}/R_{BA} (Frömter, 1972; Miller & Steinberg, 1977*a*), by a factor of ten, from 0.4 to 4.0. This could have occurred because of changes in R_{AP} and/or R_{BA}. As shown in Fig. 3, increasing apical [K^+] from 2 to 55 mM produced changes in V_{AP} that were approximately the same size in 110 mM [Na^+] solution as in 11 mM [Na^+] solution [*see also* Miller & Steinberg, 1977*a*]. This implies that R_{AP} was not greatly altered by the low [Na^+] treatment. Other experiments, not presented here, indicated that R_{BA} was reduced in low [Na^+] Ringer's solution. If this were the only effect of low [Na^+], then the transepithelial resistance, R_T, should also have decreased. In fact, it more than doubled in size, implying that R_S must have increased significantly.

The Time Course of the Fast Ouabain Response

If ouabain inhibits an electrogenic mechanism on the apical membrane, then the ouabain-induced depolarization of the apical membrane should begin with the arrival of ouabain molecules at the membrane. In order to test this hypothesis, the actual time course of the arrival of ouabain at the apical membrane would have to be measured. This measurement could not be made directly, but several experiments were designed to allow it to be made indirectly.

The time course of the arrival of ouabain, or any other substance, at the membrane depends on two different mechanisms. One mechanism is convective flow, and the other is diffusion through the unstirred layer that separates the tissue from the bulk solution (House, 1974). Convective flow establishes a new, uniform concentration of the substance in the bulk solution, and the time course of convective flow is independent of the particular substance in solution. In the present experiments, convective flow was responsible for most of the delay between the introduction of a substance and its arrival at the unstirred layer.[2] At the boundary between the bulk solution and the unstirred layer, convective flow ceases and diffusion begins. The time course of diffusion is determined by the width of the unstirred layer and the diffusion coeffcient of the substance (Crank, 1956).

Using this simple, sequential model, it was possible to infer the time course of the arrival of ouabain at the apical membrane, by comparing the response of the tissue to ouabain with its response to an increase in $[K^+]_0$. The delay due to convective flow should be equal for both ouabain and K^+, but diffusion through the unstirred layer should be

2 When a solution having a new concentration of a given substance is introduced into the perfusion system, a wavefront of this concentration change propagates toward the unstirred layer, both by convective flow and by diffusion. Convective flow, however, dominates this process. The delay due to convective flow can be estimated by dividing the volume of the chamber plus dead space (a total of 6.1 ml) by the flow rate. For flow rates between 3 and 12 ml/min, this calculation yields estimated delays ranging from 31 to 122 sec. Diffusion of the substance through the bulk solution could not significantly shorten this time, since the mean path length through the bulk solution was $\simeq 14$ mm, and it would take more than 5 hr for 1% of the change in concentration of K^+ or ouabain to diffuse this distance. Conversely, for delays in the range of 31 to 122 sec, particles of the substance in the advancing wave front could only diffuse through the bulk solution an average distance, $\bar{x}$, ranging from 170 to 340 µm, which is a negligible fraction of the total path length. This calculation is based on the relationship that $\bar{x} \simeq \sqrt{Dt}$, where D is the diffusion coefficient and t is the time (Crank, 1956), and assumes a value of D equal to 10^{-5} cm^{2}sec^{-1}, similar to that of K^+ or ouabain (Robinson & Stokes, 1968).

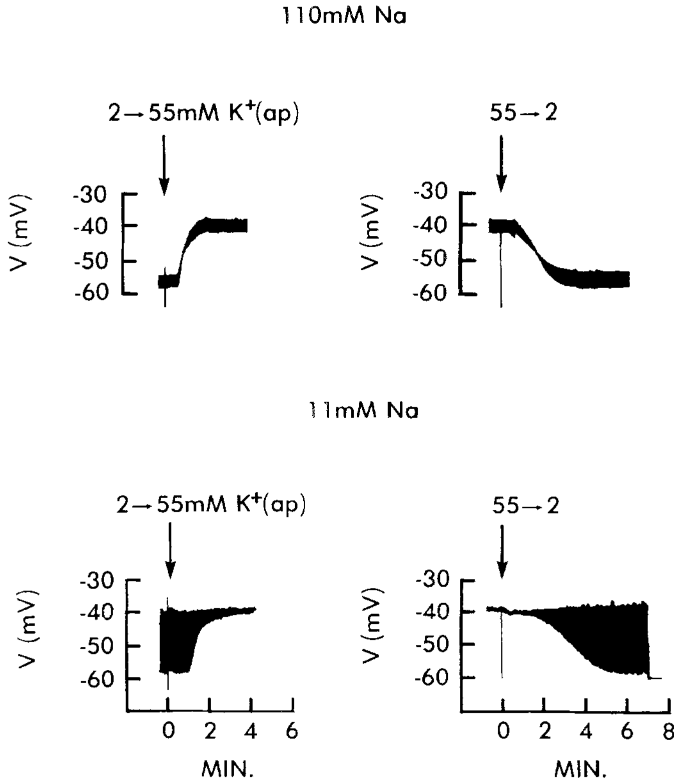

Fig. 3. Changes in V_{AP} and V_{BA} as a result of perfusion of the apical membrane with 55 mM $[K^+]$ Ringer's solution. The upper half of the figure shows the changes in V_{AP} and V_{BA} that were recorded during perfusion of both membranes with standard solutions containing 110 mM $[Na^+]$. The $[K^+]$ was elevated by an equimolar exchange of KCl for NaCl. The lower half of the figure shows similar responses that were recorded from the same cell after the tissue had been bathed on both sides for 1 hr with a Ringer's solution containing 11 mM $[Na^+]$. The $[Na^+]$ was lowered by replacing NaCl and $NaHCO_3$ by Tris Cl and Choline HCO_3, respectively. The $[K^+]$ was then increased by interchanging Tris Cl and KCl on an equimolar basis. These responses were superimposed upon a V_{AP} of $\simeq -60$ mV. This resting potential was low compared to the average value of V_{AP} (-88 mV), but the K^+-evoked changes in V_{AP} in the upper half of the figure were very similar in size and shape to those obtained in cells with larger resting potentials (Miller & Steinberg, 1977*a*). In the upper set of responses, the high $[K^+]$ solution caused the apical membrane to become depolarized with respect to the basal membrane, thereby making the TEP negative

slower for ouabain than for K^+, since the diffusion coefficient of ouabain is less than that of K^+.[3] The time of arrival of K^+ at the membrane

3 The diffusion coefficient (D) for ouabain was estimated by using the relationship that for any spherical particle, $D = KM^{-1/3}$, where K is a constant and M is the hydrated mol wt (Stein, 1967). The diffusion coefficient for K^+ is 1.7×10^{-5} cm^{2}sec^{-1} (Robinson & Stokes, 1968), and the estimated diffusion coefficient for ouabain is 0.9×10^{-5} cm^{2}sec^{-1}.

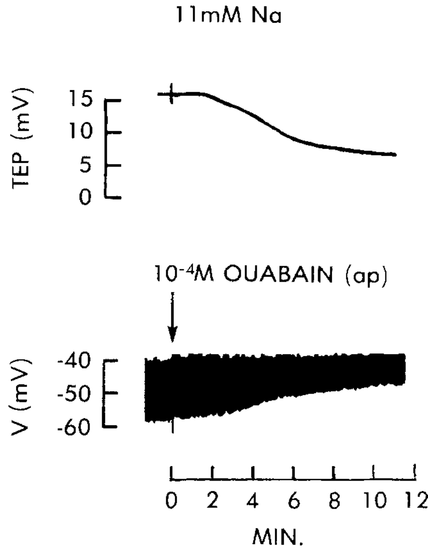

Fig. 4. Changes in V_{AP}, V_{BA}, and TEP as a result of perfusion of the apical membrane with ouabain (10^{-4} M) Ringer's solution. In contrast to Fig. 2, this tissue had been bathed on both sides for 1 hr in a Ringer's solution containing 11 mM [Na^+]. At $t=0$, the 11 mM [Na^+] solution perfusing the apical membrane was switched to one containing 10^{-4} M ouabain. These responses were superimposed upon a V_{AP} of -59 mV and a TEP of $+16$ mV, and were recorded from the same cell as the responses shown in Fig. 3

could be measured precisely with a K^+-specific microelectrode, as shown in Fig. 5.

A K^+-specific microelectrode was first positioned in the bulk solution, more than 1 mm from the apical membrane, and then the [K^+] in the apical solution was increased from 2 to 55 mM (KCl replacing NaCl). The change in the K^+-specific electrode voltage (V_{K^+}) is shown in the middle part of Fig. 5 (curve *A*), and the concomitant change in TEP is shown in the upper part of the figure (curve *A*). (The decrease in TEP resulted from a depolarization of the apical membrane.) The latency of the change in TEP was significantly longer than the latency of the change in V_{K^+}; the tissue responded with a change in TEP $\simeq 20$ sec after the increase in [K^+] was sensed by the electrode. The increase in V_{K^+}, almost instantaneous on this time scale, showed evidence of local variations in concentration as the new solution streamed past the electrode; thus, convective flow probably did not present a perfect step change in concentration to the unstirred layer. Next, the K^+-specific microelectrode was brought into contact with the apical membrane and then backed away by $\simeq 50$ µm. The [K^+] in the solution perfusing the apical

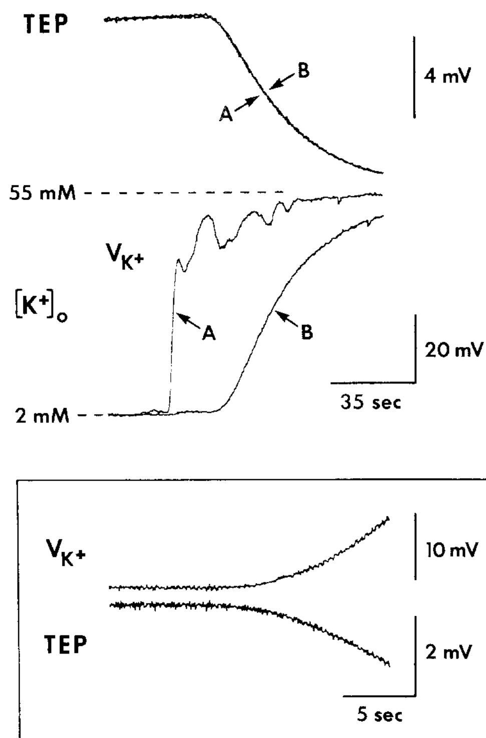

Fig. 5. Changes in TEP and V_{K^+} as a result of perfusion of the apical membrane with 55 mM $[K^+]$ Ringer's solution. The K^+-specific electrode voltage, V_{K^+}, was a measure of the logarithm of $[K^+]_o$. The two pairs of responses, labeled *A* and *B*, were recorded sequentially from the same preparation. At the beginning of these wave forms, the $[K^+]$ in the Ringer's solution entering the apical chamber was increased from 2 to 55 mM. The changes in TEP were superimposed upon a resting level of +10 mV. The tip of the K^+-specific microelectrode was either positioned more than 1 mm from the apical membrane (curves *A*), or $\simeq 50$ μm from the apical membrane (curves *B*). The inset in the lower part of the figure replots a section of curves *B* at an expanded time scale and an increased gain. The time period illustrated in the inset began $\simeq 34$ sec after the solution change

membrane was again increased from 2 to 55 mM. The pair of responses in Fig. 5, labeled *B*, showed that the latency of the decrease in TEP was similar to the latency of the build-up of K^+ at the membrane. The inset in the lower part of Fig. 5, displayed at a faster time base and higher gain, shows that these two latencies were virtually identical. Thus, the latency of the TEP response to K^+ can be used as a convenient marker, since it coincides with the onset of the build-up of K^+ at the apical membrane.

The influence of convective flow on the time of arrival of K^+ at the membrane could be assessed by increasing the flow rate. A more rapid

convective flow should reduce the latency of the change in TEP, which, as just shown, marks the onset of K^+ build-up at the membrane. The changes in TEP produced by an increase in $[K^+]$ in the apical solution from 2 to 20 mM are shown in Fig. 6*A*. These responses were recorded sequentially from the same preparation at two different flow rates, 5.0 and 10.0 ml/min. Doubling the flow rate reduced the latency of the response, from $\simeq 68$ to $\simeq 35$ sec. Assuming a step increase in concentration at the unstirred layer, and unstirred layer thickness of $\simeq 250$ μm (Miller & Steinberg, *unpublished*), diffusion through a plane sheet, an absence of convective flow across the unstirred layer (Dainty & House, 1966; Spangler & Rehm, 1968), and a diffusion coefficient for K^+ of 1.7×10^{-5} cm^{2}sec^{-1} (Robinson & Stokes, 1968), it should take $\simeq 2$ sec for a significant fraction (0.5 %) of the total change in $[K^+]_0$ to reach the apical membrane (Crank, 1956). Thus, the results in Fig. 6*A* would be expected if doubling the flow rate halved the latency due to convective flow, from 66 to 33 sec, and diffusion through the unstirred layer took 2 sec in each case. The similarity of the waveforms in Fig. 6*A* shows that, although the latency due to convective flow depends on the flow rate, the time course of the actual response (once it begins) depends on diffusion through the unstirred layer, which is independent of flow rate.

Since the diffusion coefficient for ouabain is approximately one-half that of K^+, it should take $\simeq 4$ sec for a significant fraction ($\simeq 0.5$ %) of the change in ouabain concentration to reach the apical membrane, compared to $\simeq 2$ sec for K^+. The responses to K^+ and ouabain could differ somewhat once the particles arrive at the apical membrane, since K^+ is altering an equilibrium potential and ouabain is (presumably) binding to pump sites, but this should be a second-order correction. The pump sites are probably saturated by 10^{-4} M ouabain, since 10^{-3} M ouabain does not alter the rate of the voltage response, but 10^{-5} M ouabain and lower concentrations progressively decrease this rate. The change in TEP in response to the increase in $[K^+]_0$ from 2 to 20 mM (at 10.0 ml/min) is replotted in Fig. 6*B*, at a higher gain and an expanded time scale. Superimposed upon that response is the change in TEP produced by perfusion of the apical membrane with ouabain (10^{-4} M) Ringer's solution, recorded from the same preparation at the same flow rate. The latency of the ouabain response was only several seconds longer than the latency of the K^+ response. Again, this result would be expected if diffusion through the unstirred layer took twice as long for ouabain as for K^+ (4 *vs.* 2 sec), and the latency due to convective flow was the same for both particles. These experiments demonstrate that the

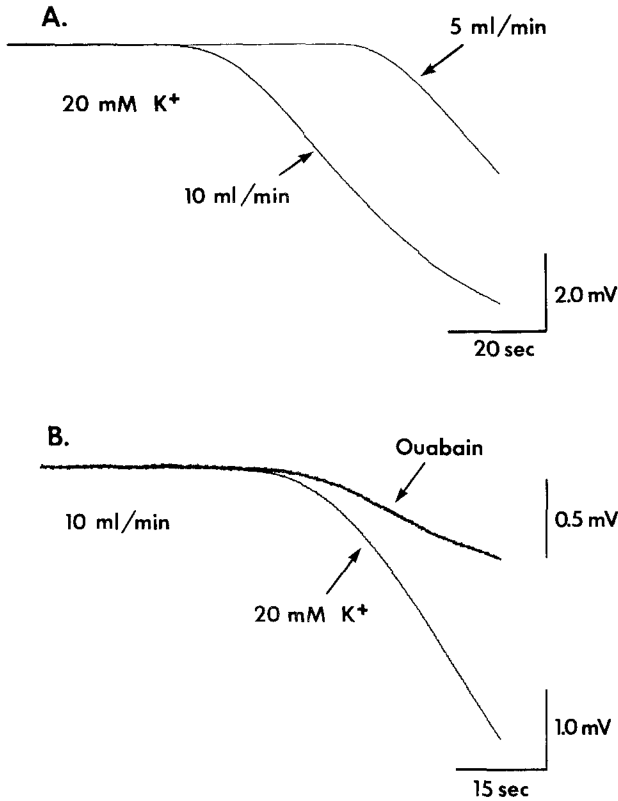

Fig. 6. Changes in TEP as a result of perfusing the apical membrane with high [K^+] or ouabain Ringer's solution. (*A*): At the start of these wave forms, the [K^+] in the solution entering the apical chamber was increased from 2 to 20 mM. The flow rate was either 5.0 or 10.0 ml/min. (*B*): The response to 20 mM [K^+] at 10.0 ml/min is replotted from *A*, at an expanded time scale and a higher gain. At the start of the wave form labeled *Ouabain*, the solution entering the apical chamber was switched from the standard Ringer's solution to ouabain (10^{-4} M) Ringer's solution. The flow rate for the ouabain response was also 10.0 ml/min. These responses were all recorded from the same preparation and were superimposed upon a TEP of +12 mV

ouabain-induced depolarization began with the arrival of ouabain molecules at the apical membrane. This result must occur if ouabain affects an electrogenic mechanism.

DNP, Cold, and Intracellular Ion Concentration Changes

There are other tests that can be made for electrogenicity. For example, dinitrophenol (DNP) should reduce the supply of ATP to the

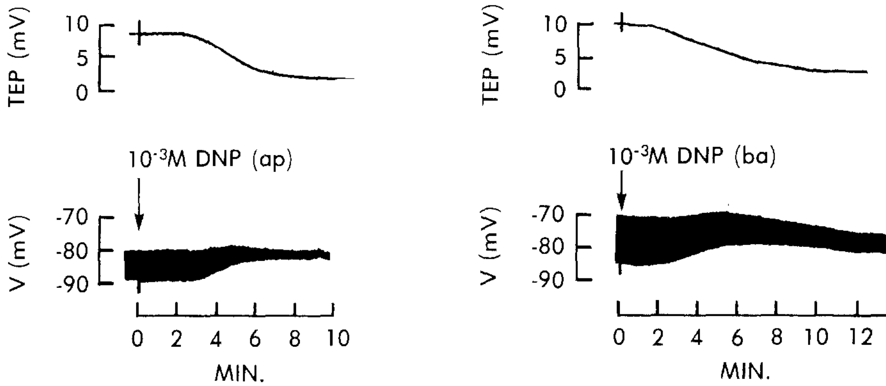

Fig. 7. Changes in V_{AP}, V_{BA} and TEP as a result of perfusion of the apical (left) or basal (right) membrane with DNP Ringer's solution. At $t=0$, the solution perfusing the apical (or basal) membrane was switched to one containing 10^{-3} M DNP. The responses on the left were superimposed upon a V_{AP} of -90 mV and a TEP of $+8$ mV. The responses on the right (recorded from a different cell) were superimposed upon a V_{AP} of -85 mV and a TEP of $+10$ mV

pump and, if the pump is electrogenic, directly alter the membrane potential. Figure 7 shows that DNP, 10^{-3} M, whether it is applied from the apical or basal solution, caused a fast depolarization of the apical membrane (lower edge of the intracellular trace).

Cooling the tissue should inhibit the pump and, if the pump is electrogenic, directly alter the membrane potential. As shown in Fig. 8, the tissue was cooled by perfusion with cold Ringer's solution, introduced at $t=0$ into the apical and basal chambers. After a latency of $\simeq 20$ sec, again due to convective flow, the cold solution arrived at the unstirred layer. The temperature change then propagated through the unstirred layer, and the apical membrane began to depolarize. The onset of the voltage change was simultaneous with the temperature change. Due to the decrease in temperature from 21 to 10 °C, the apical membrane depolarized by 13.5 mV and the TEP decreased by 7.5 mV. For an average decrease in temperature of 10 °C, the average depolarization of the apical membrane was 7.1 ± 3.4 mV ($n=5$, mean $\pm$ SEM) and the average decrease in TEP was 3.9 ± 0.3 mV ($n=10$, mean $\pm$ SEM). These changes in apical membrane potential and TEP were all much greater than would be expected if the change in temperature altered the membrane potentials solely by its effect on an RT/F electrodiffusion factor. For an average TEP of 10 mV, the predicted change would be only 0.34 mV, compared with the 3.9 mV actually observed. In Fig. 8, the predicted

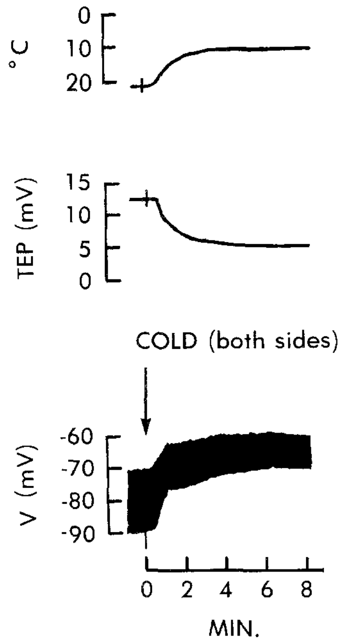

Fig. 8. Changes in V_{AP}, V_{BA} and TEP as a result of perfusion of the tissue with cold Ringer's solution. At $t=0$, the cold solutions were introduced into both the apical and basal chambers and the change in temperature (upper trace) was monitored with a thermistor in the basal chamber. These responses were superimposed upon a V_{AP} of -90 mV (lower trace) and a TEP of $+12.5$ mV (middle trace)

apical depolarization would be 3.4 mV, while 13.5 mV was actually observed. The remaining 75% of this response was most likely due to an electrogenic mechanism, which depolarized approximately 1 mV/°C. This is comparable to the changes seen in other electrogenic systems (Marmor, 1975). Prior exposure of the tissue to ouabain inhibited $\simeq 60\%$ of the response to cold, suggesting that ouabain and cold inhibit the same mechanism.

The electrogenicity of this mechanism can be tested by perfusing the tissue with 0 mM [K^+] Ringer's solution. In nerve and muscle, this treatment inhibits the Na^+-K^+ pump, and eventually the cells take up sodium, lose potassium, and depolarize (Thomas, 1972; Marmor, 1975; Glynn & Karlish, 1975). If the pump is neutral, the first effect of *reintroducing* K^+ into the apical bath would be a depolarizing diffusion potential, followed by a slow hyperpolarization as the pump restores the ionic gradients. If the pump is electrogenic and hyperpolarizing, reintroducing potassium into the apical bath would cause a rapid hyper-

polarization of the apical membrane as the pump begins to function. In order to test these predictions, the tissue was bathed for 2 hr in 0 mM $[K^+]$ Ringer's solution. This treatment had the initial effect of hyperpolarizing the apical membrane by 40 mV, from -86 to -125 mV. After $\simeq 40$ min, *both* the apical and basal membranes began to depolarize; this continued over the next 80 min until the apical membrane potential was -40 mV. Since RPE cells have a much larger conductance and a more favorable electrochemical driving force for potassium compared to sodium, this later phase most likely represented the decline of intracellular potassium ion concentration (Miller & Steinberg, 1977*a*). The standard Ringer's solution, having 2 mM $[K^+]$, was then reintroduced into the apical chamber; the apical membrane hyperpolarized as soon as the potassium ions reached the apical membrane. The initial rate of hyperpolarization was 10 mV/min (averaged over 3 min), and the total hyperpolarization was 60 mV. The speed with which this response began, its initial rate of change, and its direction, all indicate that an electrogenic mechanism had been greatly stimulated.

Discussion

The ouabain-induced depolarization of the apical membrane takes place in two phases. The fast phase coincides with the arrival of ouabain molecules at the apical membrane. The specificity of ouabain for the apical membrane and the experiments in low $[Na^+]$ solution (Figs. 3 and 4) show that the fast phase originates at the apical membrane. The ouabain specificity, by itself, strongly suggests that there is a $(Na^+ - K^+)$-ATPase on the apical membrane (Quinton *et al.*, 1973; Schwartz *et al.*, 1975; Ernst & Mills, 1977). As expected, the fast depolarization was also produced by DNP, applied from the apical or basal solution, and cold. If there is a $Na^+ - K^+$ pump located on one membrane only, then there should be a net active transport of sodium and potassium across the tissue. Conversely, the net active transport of these cations and their inhibition by apical ouabain imply a $Na^+ - K^+$ pump located on the apical membrane. In tracer experiments, it has been shown that the RPE actively transports ^{22}Na from the choroid to the retina (Miller & Steinberg, 1977*b*) and ^{42}K (^{86}Rb) in the opposite direction, from the retina to the choroid (Miller & Steinberg, *unpublished*). Both active transport systems can be inhibited by apical ouabain. The active trans-

port of potassium was also demonstrated in electrophysiological experiments, which showed that the potassium equilibrium potential, E_{K^+}, was more negative than either the apical or basal membrane batteries (Miller & Steinberg, 1977*a*; Oakley, Miller & Steinberg, 1978). Collectively, these observations indicate that the apical membrane does contain a hyperpolarizing, $Na^+ - K^+$ pump. This pump contributes approximately -10 mV to the resting potential of the apical membrane.

It could be argued that the fast effects of ouabain, apical DNP, basal DNP, and cold all resulted from a change in apical membrane conductance. This is extremely unlikely, however, since these treatments all produced different changes in membrane resistance. The changes in membrane resistance were assessed by recording the voltage divider ratio, $\Delta V_{AP}/\Delta V_{BA}$ (equal to R_{AP}/R_{BA}, Miller & Steinberg, 1977*a*). This ratio was obtained by passing constant transepithelial current pulses across the tissue and monitoring the apical and basal membrane voltage responses. In 13 experiments, ouabain increased this ratio by a small amount, 0.1 ± 0.01 (mean $\pm$SEM), from the average pre-ouabain value of 0.25 ± 0.02 (mean $\pm$SEM). In contrast, apical or basal DNP produced no consistent change in this ratio during the initial 10 min. Most importantly, there was no correlation between the size of the ouabain-induced change and the magnitude of the fast depolarization. In two experiments, for example, the changes in the voltage divider ratio were significantly different, 0.02 and 0.11, respectively, while the fast depolarizations were identical, equal to 9 mV.

Another possible explanation for the fast depolarization is that it results from a rapid accumulation of K^+ at the apical membrane, once the pump is inhibited. A straightforward calculation shows, however, that the amount of K^+ that would accumulate during a 2.5-min "fast" depolarization could diffuse away from the membrane in less than 1 msec.

The average contribution of this pump to the resting potential (-9.6 mV) coincides with the value obtained in a careful study by Gorman and Marmor on a molluscan neuron (Gorman & Marmor, 1974; Marmor, 1975). The estimate for RPE cells could be in error if, prior to inhibition, the cells were not in a steady state, having been inadvertently loaded with sodium, or depleted of potassium. This seems very unlikely, however, since the apical and basal membrane potentials remained constant over a period of hours, when the tissue was bathed on both sides with the standard Ringer's solution. The steady state was, of course, altered by ouabain, and the slow phase of membrane depolarization (27 mV/hr) was presumably due

to the Na^+ and K^+ gradients running down. A nonsteady-state condition was also produced by perfusing the tissue for 2 hr with 0 mM [K^+] Ringer's solution. Upon reintroducing the 2 mM [K^+] solution, the apical membrane hyperpolarized by 30 mV in 3 min. This was more than twice the rate of membrane depolarization during the fast phase of the ouabain response and probably represented the maximum stimulation of the pump.

The Sodium Pump and Pigment Epithelial Function

If the RPE is like other sodium transporting epithelia (for example, intestine, gallbladder, renal tubule, and choroid plexus), its sodium pump has at least two major functions. First, it maintains the cellular Na^+ and K^+ composition (and perhaps cell volume) required for normal metabolic activities; and, second, it drives fluid transport across the epithelial cell layer (Boulpaep & Sackin, 1977; Oschman, 1977; Schultz, 1977; Swanson, 1977; Wright, Wiedner & Rumrich, 1977; Diamond, 1978). The latter function is of considerable physiological significance in most, if not all, epithelia. In the RPE, such fluid transport would help maintain the composition of the ionic milieu in the extracellular space around the photoreceptors. In order to localize the active transport step responsible for fluid transport, a wide variety of epithelia have been studied autoradiographically using ^{3}H-ouabain. In almost all cases, the ($Na^+ - K^+$)-ATPase has been localized to the basal surface of the cell (Stirling, 1972; Stirling *et al.*, 1973; Stirling & Shaver, 1974; Karnaky *et al.*, 1975; Mills & Ernst, 1975; Poulsen, Bundgaard & Møller, 1975; Quinton & Tormey, 1976; Ernst & Mills, 1977; Mills, Ernst & DiBona, 1977). Striking exceptions are the frog choroid plexus (Quinton *et al.*, 1973) and the frog retinal pigment epithelium (B. Filerman & D. Bok, *private communication*), where the ATPase has been localized to the apical membrane.

The choroid plexus, like the RPE, has a net ^{22}Na transport in the basal-to-apical direction and a net ^{42}K transport in the apical-to-basal direction, both of which are inhibited by apical ouabain (Wright, 1972, 1976, 1977). The net ^{42}K transport can be increased by increasing $[K^+]_o$ from 2 to 10 mM. The sodium pump located on the apical membrane of the choroid plexus brings about a transport (from blood to CSF) of a hypertonic fluid, containing sodium, chloride, and bicarbonate (Wright *et al.*, 1977). It is not surprising that the RPE and the choroid plexus have a very similar embryological origin.

In the RPE, by analogy with the choroid plexus, it seems likely that the apical sodium pump is a major driving force for transepithelial fluid movement. The electrogenic pump current produced by the RPE (geometrical area of $0.07\,cm^2$) is approximately $3.5\,\mu A$, or 2.2×10^{13} Na^+/sec. This value was calculated from Eq. (1), using the mean value of the ouabain-induced fast potential change (9.6 mV) and the average value of R_{AP}, R_{BA}, and R_S (Miller & Steinberg, 1977*a*). If only 1/10 of this pump current, accompanied by a counterion, flowed into the extracellular space around the photoreceptors (area equal to $0.07\,cm^2$, and depth equal to 100 µm), it would replenish the sodium in that volume every 210 sec. This estimate was obtained by assuming that the fraction of the extracellular space is approximately 1% (Faber, 1969; Ogden & Ito, 1971; Zuckerman, 1973) and that the extracellular concentration of sodium is 110 mM (Sillman, Ito & Tomita, 1969; Korenbrot & Cone, 1972; Brown & Pinto, 1974). This calculation suggests that changes in sodium pump activity could exert considerable influence upon the composition of the extracellular space surrounding the photoreceptors.

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