

Effects of DIDS on the Chick Retinal Pigment Epithelium. I. Membrane Potentials, Apparent Resistances, and Mechanisms

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While little is known about the transport properties of the retinal pigment epithelium (RPE) basal membrane, mechanisms for anion movement across the basal membrane appear to be present (Miller and Steinberg, 1977; Hughes et al., 1984; Miller and Farber, 1984). This work examines the electrophysiological effects of the anion conductance blocker, 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) on the basal membrane of an *in vitro* preparation of chick retina-RPE-choroid. DIDS (10–125 μM), added to the choroidal bath, decreased the transtissue potential by decreasing the potential across the RPE. Intracellular RPE recordings showed that DIDS affected the membrane potential in 2 phases, initially hyperpolarizing the basal membrane and then, after prolonged exposure, depolarizing the apical membrane. Resistance assessment by transtissue current pulses and intracellular c-wave recordings suggested that DIDS increased basal membrane resistance (R_{ba}) during the first phase and increased apical membrane resistance (R_{ap}) during the second phase. Measurements of intracellular Cl^- activity (a_{Cl^-}) showed that Cl^- was actively accumulated by the chick RPE since it was distributed above equilibrium across both the apical and basal membranes. Perfusion of the basal membrane with 50 μM DIDS significantly increased a_{Cl^-} . The DIDS-induced basal membrane hyperpolarization, apparent increase in R_{ba} , and increase in a_{Cl^-} are all consistent with Cl^- -conductance blockade. During the second phase, apical membrane responsiveness to the light-evoked decrease in subretinal $[\text{K}^+]_o$ (Oakley, 1977) was reduced an average of 58%. This finding, given the second-phase apical membrane depolarization and apparent increase in R_{ap} , is consistent with a decrease in apical membrane K^+ conductance. This work suggests that one effect of DIDS is to block a basal membrane Cl^- conductance, while with prolonged exposure there is a secondary effect on the apical membrane, which may represent a decrease in K^+ conductance.

The retinal pigment epithelium (RPE) regulates the transport of metabolites between the photoreceptors and their choroidal blood supply. In response to illumination, communication occurs between the photoreceptors and the RPE that can be recorded in the DC electroretinogram (DC ERG) in the form of 3 components: the c-wave, the fast-oscillation trough, and the light peak (light-rise of the electrooculogram; Steinberg et al., 1985). The c-wave component originates at the RPE *apical* membrane as a voltage response to the light-evoked decrease in potassium in the subretinal space (Oakley and Green, 1976; Oakley, 1977; Steinberg et al., 1980) and is mediated by the presence of a barium-blockable potassium conductance (Griff et al., 1985). Both the fast-oscillation trough, and the light peak originate as changes in the potential of the *basal* membrane, but the mechanisms that cause these events remain unknown. In fact, little is known about the ionic conductances and transporters that contribute to the basal membrane potential in species that have these responses, i.e., reptiles, birds and mammals (Kikawada, 1968).

The RPE of frog, an amphibian, has been shown to transport anions actively from the apical side to the basal side (Hughes et al., 1984; Miller and Farber, 1984), indicating that mechanisms for anion movement must be present in the basal membrane. To investigate the significance of such anion movement in a preparation that has the 2 basal membrane light-dependent responses, we perfused the chick basal membrane with the anion transport and conductance blocker, 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS; e.g., Miller and White, 1979; Jentsch et al., 1986). We studied the effects of DIDS on the electrical properties of the RPE using intracellular recordings of membrane potentials, the membrane resistance ratio (*a* value), and RPE c-wave membrane hyperpolarizations. In addition, using double-barreled Cl^- -selective microelectrodes, we measured intracellular Cl^- activity.

This paper presents the effects of DIDS on electrical parameters of the RPE and neural retina and considers the potential mechanisms underlying these effects. The following paper (Gallemore and Steinberg, 1989) describes the effects of DIDS on responses originating at the RPE basal membrane—the light peak of the DC electroretinogram, the azide response (Linsenmeier and Steinberg, 1987), and the effects of a retinal hyperosmotic load (Shirao and Steinberg, 1987)—and addresses how these effects may be related to the mechanisms underlying these responses. Preliminary results of parts of this work have appeared in abstract form (Gallemore and Steinberg, 1987, 1988).

Materials and Methods

Preparation and solutions. White chicks (*Gallus domesticus*), 1–14 d old, were first light-adapted for at least 2 hr to promote adhesion of the

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neural retina to the RPE. They were then dark-adapted for 10 min prior to decapitation to reduce the chance of spreading depression (see below). The eye was enucleated and mounted, cornea down, in a dissecting chamber filled with a control perfusate (solutes in mM: 120.0 NaCl, 25.0 NaHCO₃, 25.0 dextrose, 5.0 KCl, 3.0 MgCl₂, 1.8 CaCl₂), which was constantly oxygenated with 95% O₂/5% CO₂ gas, pH 7.5 ± 0.1, at 36.0 ± 1.0°C. The osmolarity of this solution was 308 ± 6 mOsm (Advanced WideRange Osmometer 3W2, Advanced Instruments, Needham Heights, MA). An incision was made through the sclera, with care taken not to penetrate the choroid, and the sclera posterior to the ora serrata was dissected from the choroid. A 2–5 mm incision was made through the choroid, RPE, and retina, and a circular portion of retina-RPE-choroid tissue, 4–8 mm in diameter, was excised and placed, choroidal surface down, over a 3 mm hole in the center of a thin plastic film. The plastic film and the tissue were then mounted between 2 Lucite plates in a perfusion chamber as previously described for other preparations (Miller and Steinberg, 1977; Griff and Steinberg, 1982). The choroidal and retinal surfaces of the tissue were separately perfused such that substances added to the choroidal solution encounter the basal surface of the RPE after diffusion through the choroid, while substances added to the retinal perfusate encounter the apical surface after diffusion through the neural retina. The perfusion system was identical to those previously described except that to maintain the temperature of the perfusate at 36.0 ± 1.0°C, heater coils were inserted before the retinal and choroidal inlets of the perfusing chamber and all solutions were preheated.

The anion transport and conductance blockers DIDS and 4-acetamido-4'-isothiocyanostilbene (SITS) were added to the choroidal perfusate in the following concentrations (μM): 10.0 (DIDS only), 35.0, 50.0, 100.0, and 125.0. Care was taken to minimize exposure to white light since DIDS and SITS are light sensitive (Sigma, packing instructions).

Chick retinas are prone to spreading depression—a condition readily produced by tissue damage, low temperature, and light (Martins-Ferreira and Oliveira Castro, 1971). We reduced the frequency of spreading depression by careful dissection technique, by temperature control, by dark-adapting for 10 min following the initial period of light adaptation, and by elevating Mg²⁺ to 3.0 mM [3 times the concentration used in similar preparations of frog (Miller and Steinberg, 1977) and lizard (Griff and Steinberg, 1982)]. Because of this relatively high Mg²⁺ concentration in the control perfusate, synaptic transmission in the neural retina was normally suspended to some degree as evidenced by the small b-wave in the DC ERG (Gallemore et al., 1988).

Stimuli. Prior to the start of recording, the tissue was dark-adapted for approximately 1 hr. A halogen lamp was used to deliver a diffuse white light stimulus through a mirror, a neutral density filter and a pair of condenser lenses for a final stimulus intensity of 6×10^{-5} W/cm². To elicit a c-wave or light peak, a 4 sec or 300 sec stimulus was presented at an interval of 60–90 sec or 45–60 min, respectively.

Electrodes. Conventional microelectrodes were made from 1.0 mm glass tubing (Omega Dot Glass Co. of America, Millville, NJ) with a horizontal electrode puller (model p-77, Sutter Instruments Co., San Francisco), filled with 5.0 M potassium acetate, and beveled to a resistance of either 80–100 MΩ for intracellular recordings or 5–10 MΩ for extracellular recordings (model BV-10, Sutter Instruments Co.). Unity-gain amplifiers (model 1090, Winston Electronics, San Francisco) with input resistances of 10¹⁴ Ω, were used to measure microelectrode potentials.

Potassium- and chloride-selective microelectrodes were constructed from double-barreled glass tubing (Omega Dot Glass Co. of America), using a modification of the technique described by Djamgoz and Dawson (1986). Double-barreled glass, first broken to offset the ends of the 2 barrels, was washed in aqua regia (1 part concentrated HCl:3 parts concentrated HNO₃, vol/vol), and rinsed thoroughly in distilled water. The glass was then dried (150°C) and desiccated. Microelectrodes were pulled on the horizontal puller described above. The longer barrel was silanized for 15–45 sec by lowering it through a hole in the cap of a glass jar containing about 1 ml of dimethyl-dichloro-silane (Sigma). The reference barrel (shorter barrel) was plugged with dental wax to prevent silanization. The tip of the active barrel (longer barrel) was then filled with K⁺ liquid ion-exchange resin (Corning #477317) or Cl⁻ liquid ion-exchange resin (Corning #477913) and back-filled with 500 or 150 mM KCl. The reference barrel was back-filled with 5 M LiCl (K⁺-selective microelectrodes) or 5 M Kacetate (Cl⁻-selective microelectrodes). The tips of the K⁺-selective microelectrodes were broken to 1–3 μm by dragging the tip across the Kimwipe paper tissue. Chloride-selective microelectrodes were used without beveling and had reference barrel

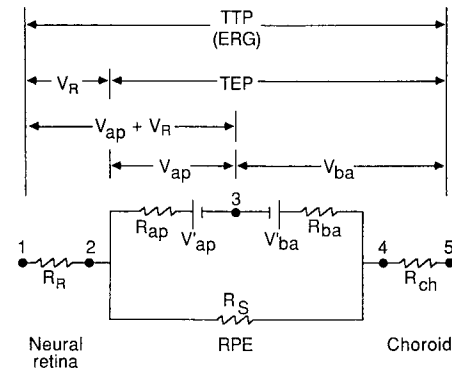


Figure 1. Equivalent circuit for the chick neural retina-RPE-choroid preparation. The neural retina and choroid are represented by the resistors R_R and R_{ch} , respectively. For the RPE, the apical membrane is represented by a resistor, R_{ap} , in series with a battery, V'_{ap} . Similarly, the basal membrane is represented by a resistor, R_{ba} , and a battery, V'_{ba} . The membrane resistances are shunted by a resistor, R_S , the paracellular pathways. As a result of current flow across R_{ap} and R_{ba} , the paracellular potentials recorded across these resistances (V_{ap} and V_{ba} , respectively) differ from the membrane batteries (V'_{ap} and V'_{ba}). The standing potential across the RPE-choroid is the transepithelial potential (TEP), and the potential across the neural retina is the transretinal potential (V_R). The trans tissue potential (TTP) is the sum of TEP and V_R . The change in TTP in response to light is the DC electroretinogram (DC ERG). Extracellular electrodes were placed in the retinal perfusate (position 1) and the choroidal perfusate (position 5). The microelectrode was placed either in the subretinal space (position 2), intracellularly in the RPE (position 3), or in the subchoroidal space (position 4).

resistances between 60 and 100 MΩ. Electrodes were connected to a high-input-impedance electrometer (WPI model F23) and calibrated before and after each experiment. Details of the calibration procedures used for each type of electrode are presented below.

Potassium-selective microelectrodes were routinely calibrated at room temperature before and after each experiment by measuring their response to a decade change in $[K^+]$: from 1 mM KCl, 111 mM NaCl to 10 mM KCl, 102 mM NaCl. The mean response of acceptable electrodes was 39 mV ($n = 20$ electrodes), similar to that reported previously (Linsenmeier and Steinberg, 1984). This is less than ideal Nernstian behavior because Na⁺ interference is most noticeable at low K⁺ concentrations. K⁺ electrodes were stable for about a week after construction.

Chloride-selective microelectrodes were calibrated in a series of solutions including 10, 25, 100, and 150 mM KCl (made isoosmotic with mannitol) at room temperature. The slope of the electrode response was determined from the regression line of voltage against the logarithm of chloride concentration ($[Cl^-]$). The slope of the electrodes used in this study averaged 55.6 ± 1.2 mV per 10-fold change in $[Cl^-]$ with a response time to 90% of the final value within 5 sec ($n = 9$ electrodes). These slopes are similar to those reported by others (Baumgarten, 1981; Saito et al., 1987). The mean active barrel resistance for these electrodes was $53 \times 10^9 \Omega$ (range, 20×10^9 to $130 \times 10^9 \Omega$). The selectivity coefficient, $k_{HCO_3/Cl}$, of the chloride electrodes for bicarbonate (HCO₃⁻), a major interfering ion that may affect the determination of intracellular chloride activity (Armstrong and Garcia-Diaz, 1980; Baumgarten, 1981), was determined by comparing the voltage observed in 25 mM KCl to that in 25 mM KHCO₃ (bubbled with 95% O₂/5% CO₂). The average value for $k_{HCO_3/Cl}$ was 0.083, corresponding to a mean Cl⁻/HCO₃⁻ selectivity of 1:12 (range, 1:10–20).

Intracellular chloride activity (a'_{Cl}) was determined from the relationship (for references see Armstrong and Garcia-Diaz, 1980):

$$a'_{Cl} = 10^{(V^{Cl}/S)}[a^o_{Cl} + (k_{HCO_3/Cl} a^o_{HCO_3})] \quad (1)$$

where V^{Cl} is the differential voltage between the ion-selective and reference barrels, S is the calibration slope of the electrode, and a^o_{Cl} and $a^o_{HCO_3}$ are the extracellular Cl⁻ and HCO₃⁻ activities, respectively, calculated from concentration assuming a Cl⁻ and HCO₃⁻ activity coefficient of 0.75 (Koryta and Stulik, 1983). The a'_{Cl} predicted for passive

distribution across each membrane was calculated using the Nernst equation and assuming that the extracellular Cl^- activity was equal to that in the bulk solution:

$$a_{\text{Cl}^-}^{\text{(passive)}} = 10^{(V_m/61 \text{ mV})} a_{\text{Cl}^-}^{\text{(active)}} \quad (2)$$

Recording configurations and equivalent circuit. The recording configurations and equivalent circuit are schematically illustrated in Figure 1. Glass microelectrodes could be placed in the subretinal space (subretinal recording, position 2), in an RPE cell (intracellular RPE recording, position 3), or in the subchoroidal space (subchoroidal recording, position 4). The subretinal recording, when referenced to the choroidal electrode (position 5), yields the transepithelial potential (TEP), and the transretinal potential (V_R) when referenced to the retinal electrode (position 1). When recording V_R in this configuration, the subretinal space is positive and the retinal bath negative. In all illustrations, however, we have inverted the V_R signal so that V_R , like TEP, is illustrated with the retinal bath as positive. Note that the transretinal and transepithelial resistances are shunted by the tissue edge resistance pathway (not illustrated), and, therefore, a change in TEP may produce a passive change in V_R , and vice versa. The intracellular recording gives the basal membrane potential (V_{ba}) when referenced to the choroidal electrode and the apical membrane potential plus transretinal potential ($V_{\text{ap}} + V_R$), when referenced to the retinal electrode. Note that, unless otherwise indicated, we have assumed that changes in ($V_{\text{ap}} + V_R$) are representative of changes in V_{ap} , neglecting changes in V_R because of their relatively small size. The potential of the retinal electrode in reference to the choroidal electrode is the transtissue potential (TTP), whose change in response to a light stimulus is defined as the DC electroretinogram.

The following equations are valid for the circuit of Figure 1, and the reader is referred to earlier publications for their derivations (Miller and Steinberg, 1977; Linsenmeier and Steinberg, 1983; Griff et al., 1985):

$$\Delta \text{TTP} = \Delta \text{TEP} + \Delta V_R \quad (3)$$

$$\Delta \text{TEP} = \Delta V_{\text{ba}} - \Delta V_{\text{ap}} \quad (4)$$

$$\Delta V_{\text{ap}} = \Delta V'_{\text{ap}} \frac{R_{\text{ba}} + R_s}{R_{\text{ap}} + R_{\text{ba}} + R_s} \quad (5)$$

$$\Delta V_{\text{ba}} = \Delta V'_{\text{ba}} \frac{R_{\text{ba}}}{R_{\text{ap}} + R_{\text{ba}} + R_s} \quad (6)$$

where TTP is the transtissue potential; TEP, transepithelial potential; V_R , voltage across the neural retina; V_{ap} (V_{ba}), measured potential of the apical (basal) membrane of the RPE; V'_{ap} , apical membrane battery; R_{ap} (R_{ba}), apical (basal) membrane resistance; R_s , paracellular shunt resistance. Equation (3) indicates that the TTP can be decreased either by a decrease in TEP, V_R , or both. The TEP represents the difference between the apical and basal membrane potentials of the RPE (Eq. 2) and, in chick, is ordinarily 8–12 mV, subretinal space positive, with the apical membrane being more hyperpolarized than the basal. Equation (4) shows that the TEP can be decreased (increased) by a basal membrane hyperpolarization (depolarization), an apical membrane depolarization (hyperpolarization), or both. Equations (5) and (6) explain the changes in membrane potential recorded at each membrane when there is a change in an apical membrane battery, $\Delta V'_{\text{ap}}$, as occurs during the RPE c-wave. Note that ΔV_{ap} is larger than ΔV_{ba} , since the potential change originates at the apical membrane, but that both ΔV_{ap} and ΔV_{ba} are smaller than the change in the battery, $\Delta V'_{\text{ap}}$. Since the TEP is defined as $V_{\text{ba}} - V_{\text{ap}}$ (Eq. 4), the RPE c-wave (TEP c-wave) will be

$$\Delta \text{TEP} = -[(R_s)/(R_{\text{ap}} + R_{\text{ba}} + R_s)] \Delta V'_{\text{ap}} \quad (7)$$

($\Delta V'_{\text{ap}}$ is negative, so ΔTEP is positive.) A change in $\Delta V'_{\text{ap}}$ and/or one or more of the resistances in Equation (7) will change the RPE c-wave amplitude.

By applying current (1.0 μA , 1.0 sec) across the tissue and measuring the appropriate voltage deflections at various locations, we could determine the following resistance values: the extracellularly recorded resistances (transretinal, R_R ; transepithelial, R_t ; transtissue, R_{tot}) and the intracellularly recorded resistance ratio (the a value, $R_{\text{ap}}/R_{\text{ba}}$). The transtissue resistance, R_{tot} , is equal to $R_R + R_t$. In our measurements, R_t also included the resistance of the choroid, R_{ch} . In 15 experiments, R_{ch} was measured with a microelectrode in the subchoroidal space referenced to the choroidal electrode and was found to be approximately 25% of

$R_t + R_{\text{ch}}$. The ratio of R_{ap} to R_{ba} is called the a value and is proportional to the voltage change (caused by the extrinsic current) across the apical and basal membranes. In calculating a , the retinal resistance was neglected because of its relatively small size, and the choroidal resistance was neglected because of its assumed constancy. By neglecting R_{ch} , the actual a values must be larger than those illustrated, and we are underestimating the absolute change in a value. Our conclusions, however, will not be affected.

All recorded signals were displayed on both a storage oscilloscope (Tektronix 5111) and a pen recorder (Brush 220) and stored on magnetic tape (Racal 4DS). Recordings were subsequently sampled and digitized (DEC PDP 11/03) and plotted on a digital X-Y plotter (Tektronix 4662).

Statistics. Numerical results are given as means \pm SEM. Student's t test was used to assess differences between paired sets of data.

Results

Effect of DIDS on the transtissue potential

When the retina-RPE-choroid preparation was perfused on its choroidal surface with 10 μM DIDS, the dark-adapted TTP decreased (Fig. 2). Since the decrease in TEP had essentially the same time course and magnitude as the decrease in TTP, we concluded that the RPE rather than the neural retina was the primary site of the effect. In this example, however, and in other sets of similar recordings, a small positive going change in the transretinal potential reduced the size of the decrease in TTP. In all experiments ($n = 22$), and for all concentrations of DIDS, this change in transretinal potential was not more than 10% of the change in TEP. DIDS had a prominent effect on the TTP even at the lowest concentration used, 10 μM (Fig. 2). Higher concentrations (35–125 μM) produced faster and larger decreases in the TTP, while recovery times were significantly prolonged; the maximal effect of choroidal DIDS was produced with concentrations of 100 μM and greater. We also used a related stilbene derivative, SITS, in concentrations of 35, 50, 100, and 125 μM . Both SITS and DIDS had similar effects on the electrical properties of the chick RPE, decreasing the TTP, increasing tissue resistance, and decreasing the c-wave, as we will describe. Since DIDS was more potent (Gallemore and Steinberg, 1989), we limited our intracellular experiments to the use of DIDS.

Figure 3 illustrates V_{ap} and V_{ba} during choroidal perfusion with 50 μM (Fig. 3A) and 100 μM (Fig. 3B) DIDS. Although the decrease in TTP appeared to be monophasic, membrane potential changes occurred in 2 distinct phases as indicated by the vertical interrupted lines. In the *first phase*, during the initial 5–10 min, both membranes hyperpolarized, but the basal membrane hyperpolarization was larger and faster than the apical membrane hyperpolarization. This is consistent with choroidal DIDS hyperpolarizing the basal membrane, while the smaller apical membrane hyperpolarization is likely due to shunting of the basal membrane potential through the paracellular resistance (R_s in Fig. 1). In the *second phase*, which occurred after 5–10 min, the apical membrane depolarized. In general, this depolarization was subtle, as shown in Figure 3A (50 μM DIDS). With higher concentrations of DIDS, however, this depolarization could be quite prominent, as shown in Figure 3B (100 μM DIDS). Similar results were obtained in 5 experiments (50 μM choroidal DIDS, $n = 2$, and 100 μM choroidal DIDS, $n = 3$). In summary, the data are consistent with choroidal DIDS initially hyperpolarizing the basal membrane and then, following prolonged exposure, depolarizing the apical membrane.

Resistance measurements: R_t and the a value

Choroidal DIDS also caused a large and reversible increase in the transtissue resistance, R_{tot} (Fig. 4A) that was due to a 12.0

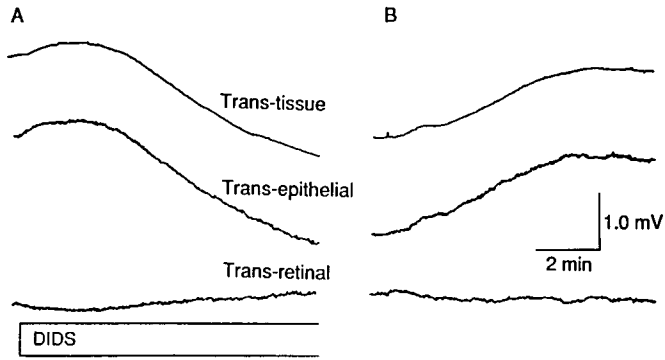


Figure 2. Effect of choroidal DIDS perfusion ($10 \mu\text{M}$) on the TTP and its transepithelial and transretinal components. In *A*, the perfusate was changed to the test solution containing DIDS as indicated at the bottom by the bar. The recovery of the TTP in *B* occurred 40 min after return to normal Ringer from the DIDS solution.

$\pm 1.6\%$ ($n = 7$) increase in the RPE resistance (R_r , or more precisely, $R_r + R_{ch}$) and not to an increase in retinal resistance (R_R). Since R_{ch} is approximately 25% of $R_r + R_{ch}$, the change in R_r itself was quite large, about 16%. Note that the 50% decrease in R_R in this experiment was unusually large, compared with the average of $18.5 \pm 3.5\%$ ($n = 7$).

The DIDS-induced increase in R_r could have resulted from an increase in R_{ap} , R_{ba} , or R_s (see Fig. 1). As shown in Figure 5*B*, although the increase in R_{tot} (and thus R_r as well) appeared to be monophasic, choroidal DIDS altered the ratio of apical to basal membrane resistances (a value) in 2 distinct phases. In the *first phase*, concordant with basal membrane hyperpolarization (Fig. 2), the a value decreased while R_{tot} increased, consistent with an increase in RPE basal membrane resistance. In the *second phase*, concordant with apical membrane depolarization, the a value increased, while R_{tot} continued to increase, consistent

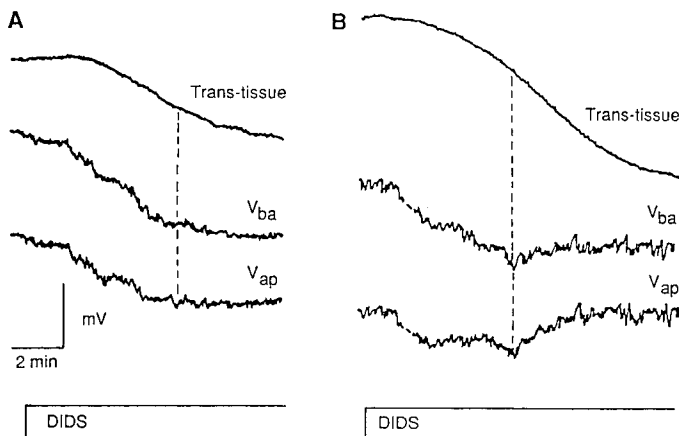


Figure 3. Effect of choroidal DIDS perfusion on RPE membrane potentials and the TTP for $50 \mu\text{M}$ DIDS (*A*) and $100 \mu\text{M}$ DIDS (*B*). The TTP was recorded differentially between the retinal and choroidal perfusates. Simultaneously, the apical membrane potential (V_{ap}) was recorded differentially between the intracellular microelectrode and the retinal perfusate; the basal membrane potential (V_{ba}) was recorded between the microelectrode and the choroidal perfusate. Although the TTP decrease appears to be monophasic, the effect of DIDS on the RPE membrane potentials occurred in 2 distinct phases, which are demarcated by the vertical interrupted lines. The perfusate was changed to the test solution containing DIDS as indicated at the bottom by the bars. Vertical calibration: 2 mV for V_{ap} and V_{ba} and 1 mV for trans-tissue.

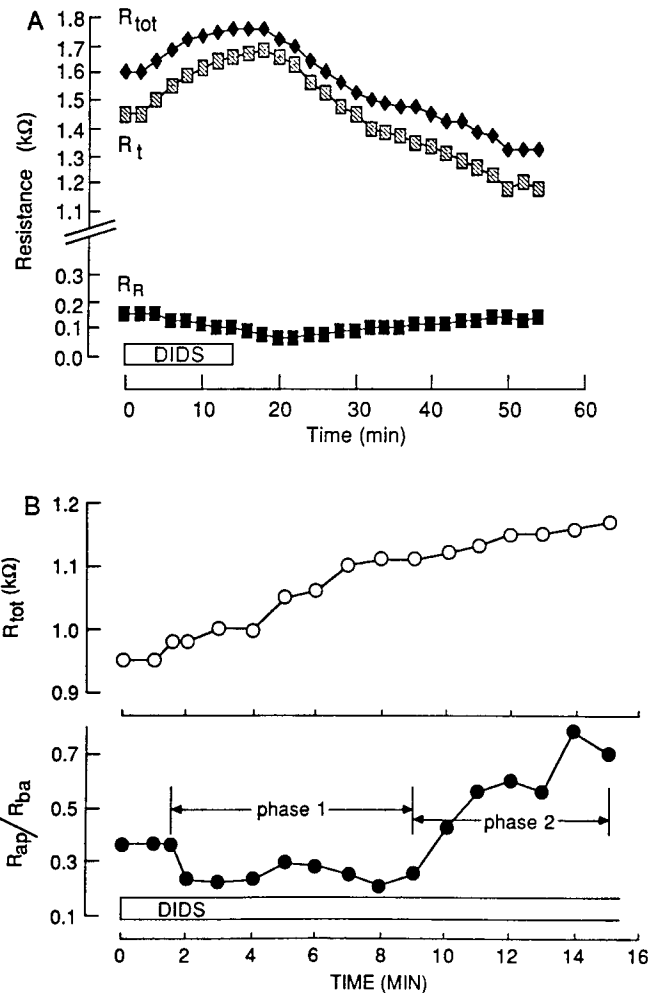


Figure 4. Effect of choroidal DIDS perfusion on resistance measurements. *A*, Change in the total tissue resistance (R_{tot}) and its components, the transepithelial resistance, R_t , and the transretinal resistance, R_R , before, during, and after choroidal perfusion with $35 \mu\text{M}$ DIDS. DIDS caused an increase in R_{tot} that was due to an increase in R_t . Note the 50% decrease in R_R . *B*, Effect of choroidal DIDS perfusion ($100 \mu\text{M}$) on the intracellularly recorded resistance ratio (R_{ap}/R_{ba} , or a value) as correlated with changes in total tissue resistance, R_{tot} . Although the increase in R_{tot} appears to be monophasic, the effect of DIDS on the a value actually occurred in 2 phases: an early phase (Phase 1) and a later phase following prolonged exposure to DIDS (Phase 2).

with an increase in RPE apical membrane resistance. In this example, the a value increased in the second phase above the a value before choroidal perfusion with $100 \mu\text{M}$ DIDS. In other cases, the a value did not increase in the second phase above its pre-DIDS value (3 cells: $50 \mu\text{M}$, $n = 1$; $100 \mu\text{M}$, $n = 2$).

There is a second method, the intracellular recording of the c-wave of the DC ERG, which is independent of the a -value measurement and can be used to identify the origin of the DIDS-induced increase in R_r . Before considering the results of this analysis, we must first describe the effects of choroidal DIDS on the c-wave.

C-wave

A reversible decrease in c-wave amplitude was always observed during choroidal DIDS perfusion (Fig. 5). The voltage changes of the c-wave are produced by a light-evoked decrease in sub-retinal K^+ (Oakley and Green, 1976; Oakley, 1977; Steinberg

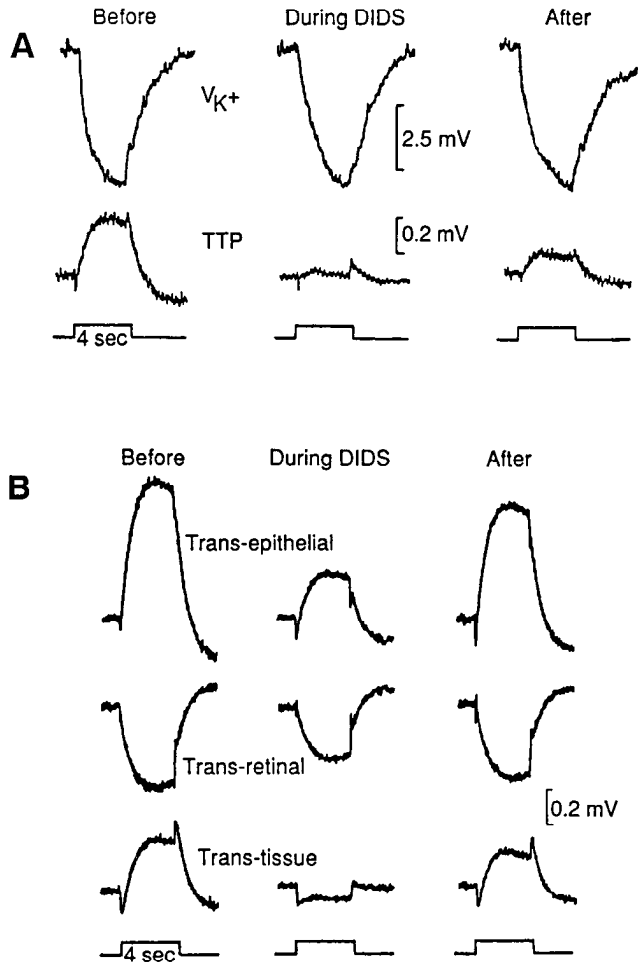


Figure 5. Choroidal DIDS perfusion and the c-wave. *A*, Light-evoked changes in subretinal potassium concentration (V_{K^+}) and the transstissue c-wave (TTP) in response to a 4 sec stimulus (6×10^{-5} W/cm²) before, during, and after choroidal perfusion with 50 μ M DIDS. A 10.0 mV change in V_{K^+} corresponds to a change of approximately 2.5 mM in the extracellular K^+ level in the subretinal space, $[K^+]_o$. The dark-adapted $[K^+]_o$ was measured to be 5.4 mM, 0.4 mM greater than the control perfusate, and was not changed by DIDS (not shown). *B*, Selected trans-epithelial (RPE c-wave) and transretinal (slow PIII) components of the TTP c-wave before, during, and after choroidal perfusion with 35 μ M DIDS.

et al., 1980). Figure 5*A* illustrates the light-evoked K^+ decrease measured when the c-wave reduction in DIDS was maximal ("During DIDS") compared with control and recovery responses. The light-evoked K^+ decrease, recorded with a K^+ -selective microelectrode in the subretinal space, was not reduced at any time by choroidal DIDS and, therefore, did not cause the c-wave reduction. Similar results were obtained in 3 tissues (50 μ M DIDS).

The c-wave amplitude decrease and recovery paralleled in time course the decrease and recovery of the TTP, with the c-wave amplitude reaching its minimum at about the minimum of the TTP. Figure 6 shows that for all concentrations of choroidal DIDS, the maximum decrease in c-wave amplitude was well correlated with the size of the TTP (standing potential) change. These data suggest, therefore, that the mechanisms underlying the changes in the c-wave and TTP are related.

The transstissue c-wave is the sum of the negative-going potential generated in the neural retina (slow PIII) and a positive-

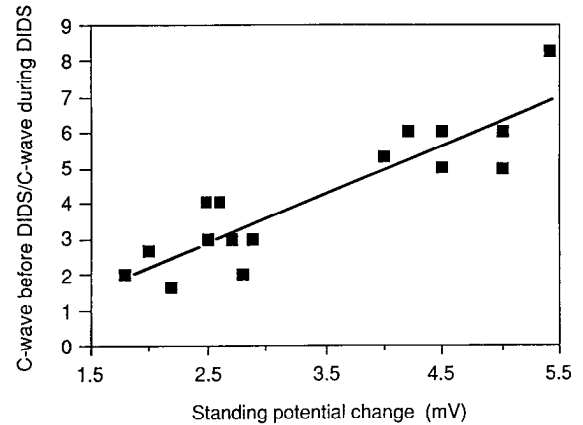


Figure 6. Maximal change in c-wave amplitude (c-wave during DIDS/c-wave before DIDS) as a function of the maximal change in transtissue potential (standing potential) caused by DIDS. Data from choroidal perfusions with DIDS (10–125 μ M) in 21 chicks are shown. The line is a linear regression with a slope of 1.39, a y -intercept of 1.50 and a correlation coefficient of 0.80.

going component from the RPE, the transepithelial or RPE c-wave (Faber, 1969; Rodieck, 1972; Linsenmeier and Steinberg, 1983). The change in c-wave amplitude could have resulted from a change in either or both of these components. The transepithelial and transretinal c-wave components can be separately recorded by positioning a microelectrode in the subretinal space. We found that the RPE c-wave became significantly smaller in amplitude while slow PIII decreased much less,¹ for example, 67 versus 38% in Figure 5*B*. Since a decrease in slow PIII alone would increase the c-wave, the decrease in the trans-tissue c-wave must be attributed to the decrease in its RPE component.

Resistance assessment: intracellular c-wave responses

Figure 7 shows apical and basal membrane c-wave hyperpolarizations and the transstissue c-wave in response to 4 sec flashes presented before and during choroidal perfusion with 50 μ M DIDS. As described above, the RPE c-wave results from a hyperpolarization of the apical membrane in response to a light-evoked decrease in subretinal potassium. The apical membrane hyperpolarization is shunted to the basal membrane (across R_r , Fig. 1), so that both the apical and basal membranes hyperpolarize, and this is illustrated in the control response in Figure 7*A* (V_{ap} does not include V_r in this figure²). Note that the apical membrane hyperpolarization is larger than the basal membrane

¹ Effect of DIDS on the transretinal potential (Fig. 2) and slow PIII were not due to changes in subretinal $[K^+]_o$. Dark-adapted subretinal $[K^+]_o$ was not altered by choroidal DIDS (50 μ M, $n = 3$) and the light-evoked decrease in $[K^+]_o$, which generates slow PIII (Witkovsky et al., 1975; Wen and Oakley, 1987), was not affected by DIDS (Fig. 5*A*). At least 2 factors contribute to the decrease in slow PIII. First, a fraction of slow PIII is a voltage drop across the transretinal (R_r) resistance produced by the RPE c-wave current (Linsenmeier and Steinberg, 1987), so that a decrease in the RPE c-wave alone will decrease slow PIII. Second, the decrease in R_r during choroidal DIDS perfusion (Fig. 4*A*) will decrease slow PIII. The increase of the dark-adapted transretinal potential may represent a passive voltage drop across the transretinal resistance of the decreasing TEP.

² With the intracellular microelectrode referenced to the retinal bath, we actually measure ($V_{ap} + V_r$) (see Materials and Methods and Fig. 1). To estimate V_{ap} more accurately, we subtracted the change in V_r during illumination (slow PIII) using data from a similar experiment (controlling for DIDS concentration, maximum percent decrease in TTP c-wave, control TTP, and control c-wave amplitude). We found that our conclusions were the same whether we looked at the change in ($V_{ap} + V_r$) or the estimated change in V_{ap} . We have illustrated the estimated change in V_{ap} in Figure 7.

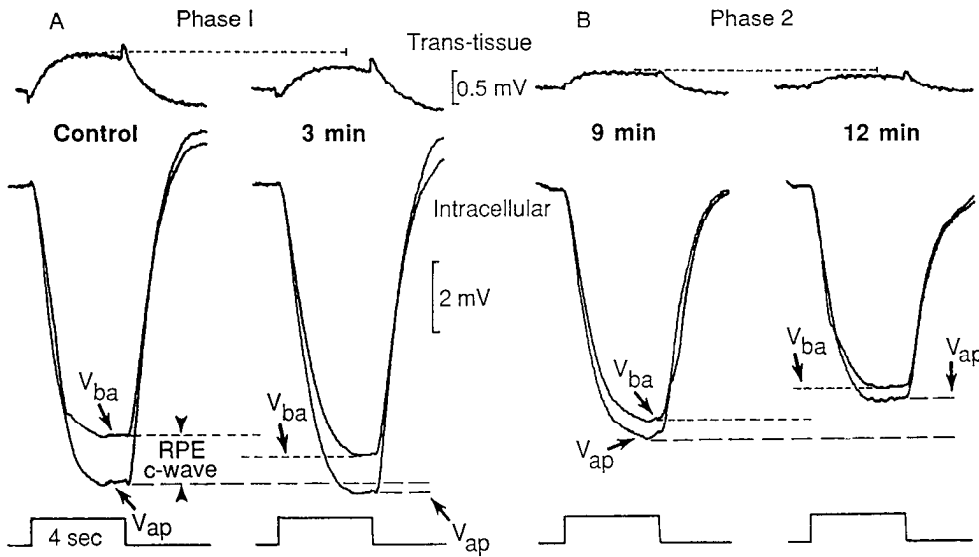


Figure 7. Effect of 50 μM choroidal DIDS on the intracellularly recorded RPE c-wave as correlated with changes in the transtissue c-wave. The recording configuration is the same as in Figure 3. The control response illustrates that the RPE c-wave is the difference between the apical (V_{ap}) and basal (V_{ba}) membrane hyperpolarizations in response to light. The effect of choroidal DIDS on the membrane hyperpolarizations occurred in 2 distinct phases: (A) an earlier phase (Phase 1) where both apical and basal hyperpolarizations increased and (B) a later phase (Phase 2) where both apical and basal hyperpolarizations decreased. Note decrease in transtissue c-wave during both phases. The times following the start of DIDS perfusion are indicated in the figure.

hyperpolarization and that the difference between these hyperpolarizations is the RPE c-wave.

The RPE c-wave hyperpolarizations also changed in 2 phases. In the *first phase* (Fig. 7A), concordant with the underlying basal membrane hyperpolarization (Fig. 3), the c-wave hyperpolarizations of both V_{ap} and V_{ba} increased, with the increase in V_{ba} being larger so that a decrease in the transtissue c-wave occurred. Since the light-evoked K^+ decrease was unchanged by DIDS (Fig. 5A), the intracellular c-wave hyperpolarizations could have been altered by a change in R_{ap} , R_{ba} , or R_s (see Eqs. 5 and 6). The a -value and R_i measurements (Fig. 4B) pointed to an apparent increase in R_{ba} during phase 1. If R_{ba} increased, then Equations (5) and (6) predict *larger* c-wave hyperpolarizations of V_{ap} and V_{ba} , with different predictions for changes in R_{ap} or R_s (Linsenmeier and Steinberg, 1983). The larger c-wave hyperpolarizations in phase 1 of Figure 7, therefore, support the hypothesis that R_{ba} increased. As predicted by Equation (7), an apparent *increase* in R_{ba} alone can reduce the amplitude of the c-wave. This finding also has been reported for the effect of basal hyperosmolarity on the c-wave (Shirao and Steinberg, 1987).

In the *second phase* (Fig. 7B), concordant with apical membrane depolarization, the c-wave changes in both V_{ap} and V_{ba} were smaller, with the decrease in V_{ap} being greater so that c-wave amplitude decreased further. Resistance measurements during phase 2 (Fig. 4B) suggested that R_{ap} increased during this period. Consistent with this result, Equations (5) and (6) predict *smaller* c-wave hyperpolarizations when R_{ap} increases. Note that the apical membrane hyperpolarization became significantly smaller during the second phase (compare with control response) and by 16 min (not shown) had increased by 45% as compared to the control response. Similar results were observed in all 4 experiments (50 μM DIDS, $n = 2$; 100 μM DIDS, $n = 2$; range of maximum percent decrease in apical membrane hyperpolarization: 45–70%; mean = 57.5%). This indicates that the responsiveness of the apical membrane to a given change in $[\text{K}^+]_o$ had decreased significantly. A decrease in the apical membrane potassium conductance (g_{K^+}) would account for the apparent phase 2 increase in R_{ap} as well as the phase 2 apical membrane depolarization (Griff et al., 1985).

Intracellular chloride activity in chick RPE

In other systems, DIDS has been shown to block Cl^- channels (Miller and White, 1979; Nelson et al., 1984; Inoue, 1985; Kimmich and Montrose, 1985). If DIDS hyperpolarizes the RPE basal membrane and increases its resistance by blocking a Cl^- conductance, then we would predict that $a_{\text{Cl}^-}^i$ is above equilibrium in the RPE cell and that application of DIDS to the basal membrane should increase $a_{\text{Cl}^-}^i$. To test this possibility, we measured $a_{\text{Cl}^-}^i$ in chick RPE with double-barreled Cl^- -selective microelectrodes.

Criteria for acceptable impalements included (1) an abrupt negative deflection of V_{ap} , V_{ba} , and V_{Cl^-} on penetration of the cell, (2) a stable intracellular potential for at least 30 sec, and (3) return of the electrical potential to within 2 mV of baseline upon withdrawal of the electrode (Armstrong and Garcia-Diaz, 1980). All penetrations were made across the apical membrane. Note that our calculations of passive distribution of Cl^- activity assume the same extracellular Cl^- activity as the bulk solution. Our conclusions would be unaltered even if the extracellular Cl^- activity values were within ± 10 mM of the bulk solution value.

Because the calculation of $a_{\text{Cl}^-}^i$ requires an accurate measurement of membrane potential, and the larger tip size of the double-barreled microelectrode might cause significant membrane damage, we first compared measurements of V_{ap} and V_{ba} made with conventional single-barreled electrodes versus similar measurements made with double-barreled Cl^- -selective electrodes. For 13 cells in 9 tissues impaled with conventional microelectrodes, $V_{ap} = -70 \pm 1.8$ mV and $V_{ba} = -63 \pm 2.1$ mV (not significantly different from those recently reported; Shirao and Steinberg, 1987). For 9 cells in 4 tissues impaled with Cl^- -selective microelectrodes, $V_{ap} = -67 \pm 4.0$ mV and $V_{ba} = -60 \pm 3.7$ mV. Though lower, these values do not differ significantly from those measured with conventional microelectrodes ($p > 0.4$ for V_{ap} and $p > 0.3$ for V_{ba}), indicating that significant membrane damage was not a complication.

Figure 8A illustrates a representative recording of V_{ap} , V_{ba} , and V_{Cl^-} . To withdraw from the cell the electrode was retracted only several microns to prevent penetration of the neural retina,

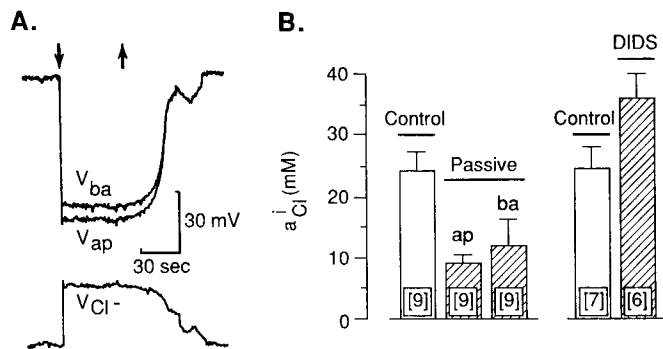


Figure 8. Intracellular chloride activity in chick RPE. *A*, Representative recording of V_{ap} , V_{ba} , and V_{Cl^-} with a double-barreled Cl^- -selective microelectrode. For this cell, $V_{ap} = -73$ mV, $V_{ba} = -64$ mV, and $a_{Cl^-} = 20.1$ mM. The predicted a_{Cl^-} 's for passive distribution of Cl^- across the apical and basal membranes are 6.7 and 9.4 mM, respectively. Arrows demarcate time of microelectrode penetration and withdrawal across the RPE apical membrane (see text for details). *B*, a_{Cl^-} is distributed above equilibrium across both the apical and basal membranes. The average a_{Cl^-} (9 cells in 4 tissues) was 24 ± 2.9 mM, about 2.6 times the a_{Cl^-} predicted for passive distribution across the apical membrane and 2.0 times the a_{Cl^-} predicted for passive distribution across the basal membrane. Perfusion of the basal membrane with $50 \mu M$ DIDS produced a significant increase in a_{Cl^-} ($p < 0.05$). In the presence of basal DIDS, the average a_{Cl^-} (6 cells in 2 tissues) was 36 ± 4.1 mM, about 1.5 times the average a_{Cl^-} measured in control perfusate (for 7 control cells in the 2 tissues, $a_{Cl^-} = 24.5 \pm 3.5$ mM).

and, thus, the cell was slowly lost, as indicated by the slow recovery of potentials. For this cell, $V_{ap} = -73$ mV, $V_{ba} = -64$ mV, and $a_{Cl^-} = 20.1$ mM. Using Equation (2), the predicted a_{Cl^-} 's for passive distribution of chloride across the apical and basal membranes are 6.7 and 9.4 mM, respectively. Thus, Cl^- is actively accumulated into the chick RPE cell.

As shown in Figure 8*B*, additional measurements confirmed that a_{Cl^-} was distributed above equilibrium across both the apical and basal membranes. The average a_{Cl^-} (control, left; 9 cells in 4 tissues) was 24 ± 2.9 mM, about 2.6 times the a_{Cl^-} predicted for passive distribution across the apical membrane and 2.0 times the a_{Cl^-} predicted for passive distribution across the basal membrane (cross-hatched, left). Also shown in Figure 8*B*, perfusion of the basal membrane with $50 \mu M$ DIDS produced a significant increase in a_{Cl^-} ($p < 0.05$). In the presence of basal DIDS (12–15 min exposure), the average a_{Cl^-} (cross-hatched, right; 6 cells in 2 tissues) was 36 ± 4.1 mM, about 1.5 times (or 50% more than), the average a_{Cl^-} measured in control perfusate (control, right; for 7 control cells in the 2 tissues, $a_{Cl^-} = 24.5 \pm 3.5$ mM). In 2 experiments, DIDS applied to the choroidal bath did not significantly change the apparent a_{Cl^-} outside the RPE apical membrane (not shown).

Discussion

Perfusion of the RPE basal membrane with micromolar concentrations of DIDS had significant effects on the chick RPE preparation. DIDS decreased the TTP, reduced the amplitude of the c-wave of the DC ERG and increased the total tissue resistance (R_{tot}). The effects of DIDS on the RPE occurred in 2 distinct phases. In the *first phase*, the basal membrane hyperpolarized and basal membrane resistance appeared to increase. In the *second phase*, which followed prolonged exposure, the apical membrane depolarized and its resistance appeared to increase.

Phase 1: effect on the basal membrane

The effects of Phase 1 are consistent with DIDS decreasing the conductance of some ion channel at the basal membrane. The ion permeating this channel must have an equilibrium potential (E_{ion}) more depolarized than the resting potential of the basal membrane (E_m) in order to hyperpolarize it when blocked. E_m for the basal membrane was -63 ± 2.1 mV, and this practically eliminates a K^+ conductance (g_{K^+}) since E_{K^+} should be more hyperpolarized than E_m and, therefore, a decrease in g_{K^+} would depolarize the membrane (Miller and Steinberg, 1977; La Cour et al., 1986). On the other hand, either a decrease in a Cl^- conductance (g_{Cl^-} where Cl^- is not passively distributed) or Na^+ conductance (g_{Na^+}) could hyperpolarize E_m . Since DIDS has been found to decrease g_{Cl^-} in some systems (Miller and White, 1979; Nelson et al., 1984; Inoue, 1985; Kimmich and Montrose, 1985) and has not, to our knowledge, been found to affect g_{Na^+} , this analysis is consistent with basal DIDS hyperpolarizing the RPE basal membrane by reducing a Cl^- conductance.

An effect of DIDS on a g_{Cl^-} at the basal membrane could be direct, i.e. by blocking Cl^- channels, or indirect, i.e., by blocking a Cl^- uptake transporter, decreasing $[Cl^-]_i$, and thereby secondarily causing a decrease in g_{Cl^-} . The finding that DIDS increases a_{Cl^-} (Fig. 8*B*) is most consistent, therefore, with a direct effect of DIDS on a Cl^- conductance at the basal membrane. There is a precedent for direct Cl^- conductance blockade by DIDS in other systems (Miller and White, 1979; Nelson et al., 1984; Inoue, 1985; Kimmich and Montrose, 1985). In many preparations, however, DIDS is also known to block a number of other anion transport mechanisms (see, for example, Jentsch et al., 1986; Hughes et al., 1987; Knicklebein et al., 1985; L'Alleman et al., 1985; Sasaki et al., 1987). Thus, although our results are most easily explained by blockade of a basal membrane g_{Cl^-} , other mechanisms could contribute to the DIDS effects.

Finally, that the RPE actively transports Cl^- from the apical side to the basal can only be understood in terms of an apical membrane Cl^- uptake mechanism and a basal membrane Cl^- permeation mechanism in frog (Miller and Steinberg, 1977), dog (Tsuboi, 1987), bovine (Edelman and Miller, 1987), and embryonic chick (Frambach and Misfeldt, 1983). Our measurements of a_{Cl^-} in chick RPE (Fig. 8) indicate that Cl^- is distributed above equilibrium across both the apical and basal membranes. This indicates that our preparation also actively transports Cl^- , although no directionality can be inferred. Assuming that in chick net Cl^- transport is also directed apical to basal, our results suggest the presence of a DIDS-sensitive chloride conductance through which Cl^- , at least in part, is secreted. Edelman et al. (1988) recently found that basal DIDS blocks net Cl^- transport across the frog RPE, which is also consistent with a basal membrane DIDS-sensitive Cl^- secretory mechanism.

The ion-exchange resin used in our Cl^- -selective electrodes has been shown to be sensitive to stilbene derivatives such as DIDS (Chao and Armstrong, 1987). The interference of *extracellular* DIDS in our measurements should be insignificant since RPE cells were penetrated across their apical membrane while the basal membrane was perfused with DIDS. In addition, DIDS applied to the choroidal bath did not significantly change the apparent a_{Cl^-} outside the RPE apical membrane. Regarding *intracellular* DIDS, it and similar compounds have been characterized as "nonpenetrating probes" (Cabantchick and Roth-

stein, 1972; Grinstein et al., 1979). Assuming, however, that any presumed permeability of the RPE membrane to DIDS is entirely passive, we can estimate the maximal interference of intracellular DIDS.³ If this interference was present in our measurements, then the apparent increase in a_{Cl}^- produced by choroidal perfusion with 50 μ M DIDS would be 20%, rather than 50% (see Results). This error does not alter the conclusions we have drawn.

Phase 2: effects on the apical membrane

How might DIDS, following prolonged perfusion of the basal membrane, depolarize the apical membrane and produce an apparent increase in R_{ap} ? This effect may be indirect. By blocking a basal membrane transport mechanism and/or conductance, DIDS might change the intracellular activity of some ion, for example, Cl^- , which would then affect a mechanism at the apical membrane, sensitive to the intracellular activity of that ion. Alternatively, DIDS could diffuse to the apical membrane and act directly on a DIDS-sensitive mechanism. In frog RPE, a DIDS-sensitive apical membrane electrogenic Na^+ -dependent HCO_3^- cotransporter has recently been described (Hughes et al., 1987). Inhibition of this transporter with DIDS applied extracellularly to the apical membrane depolarized the apical membrane. We have found, as well, that application of DIDS to the apical side of the chick preparation also depolarizes the apical membrane (2–3 mV; $n = 3$). Application of DIDS to the basal membrane first, however, did not block the apical membrane depolarization produced by apical DIDS. Furthermore, application of apical DIDS first did not block the second phase reduction of the RPE c-wave hyperpolarizations produced by basal DIDS (Gallemore and Steinberg, unpublished observations). Taken together, these results suggest that a direct effect of DIDS on the apical membrane is not the mechanism of the effects observed during the second phase.

During the second phase, apical membrane responsiveness to the light-evoked decrease in K^+ was reduced an average of 57.5%. A reduction in g_{K^+} at the apical membrane would depolarize that membrane and increase its resistance (Griff et al., 1985). These results suggest that prolonged exposure of the basal membrane to DIDS can reduce the potassium conductance of the apical membrane. If basal DIDS decreases apical g_{K^+} , then this effect would most likely be indirect since, to our knowledge, DIDS has not been found to decrease directly a g_{K^+} in any system studied.⁴ Recently, the RPE apical membrane g_{K^+} was found to be pH sensitive (Joseph and Miller, 1986; Keller et al., 1986). Since $[Cl^-]_i$ is involved in the regulation of intracellular pH (for a review, see Roos and Boron, 1981), basal DIDS may affect a pH-sensitive apical membrane potassium conductance by its effect on intracellular Cl^- activity.

In sum, we have presented evidence in support of a DIDS-inhibitable basal membrane Cl^- conductance. With prolonged exposure to basal DIDS an effect on the apical membrane was

observed that appears to involve the reduction of a K^+ conductance.

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³ Given a mean RPE basal membrane potential of -63 mV, for an extracellular DIDS concentration of 50 μ M, the equilibrium intracellular concentration of DIDS (calculated from the Nernst equation) cannot be more than 20% of this, or 10 μ M (DIDS has 2 negative charges). Given a maximum electrode selectivity of Cl^- : DIDS of 1:1000, and an activity coefficient of 0.75 for DIDS, this would cause an increase of 7.5 mM in the apparent a_{Cl}^- .

⁴ Inoue (1986), however, found that in the squid giant axon, DIDS increased a potassium conductance. In addition, Biagi (1986) demonstrated that the stilbene derivative SITS had secondary effects on a g_{K^+} in proximal tubule. In this case, g_{K^+} increased as well.

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