ISOPOTENTIALITY AND AN OPTICAL DETERMINATION OF SERIES RESISTANCE IN *LIMULUS* VENTRAL PHOTORECEPTORS

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SUMMARY

1. Photoreceptor somas in the ventral rudimentary eye of *Limulus polyphemus* were impaled with three micropipettes. Two micropipettes were connected in a voltage-clamp circuit and the cells were stimulated by brief flashes. The third micropipette did not measure any significant deviations from the 'clamped' voltage during responses to the flashes, in several geometries of electrode placement, even for very bright flashes. Therefore using the described techniques there is no evidence for spatial non-uniformity of intracellular voltage in the soma of these photoreceptors.

2. A voltage-sensitive dye was used to monitor light-induced changes in membrane voltage while intracellular voltage was held clamped by a feed-back circuit. With a known series resistance connected between the bath and ground the dye recorded a light-induced change in membrane voltage. When there was no added series resistance, the light-induced change was smaller and often undetectable. From these data the naturally occurring series resistance was calculated to be $\leqslant 30$ kΩ.

3. From these measurements, as well as from calculations for a model spherical cell, we conclude that membrane potential can be controlled to within 2 mV using our micropipette 'point clamp' methods, for all but the brightest stimuli.

INTRODUCTION

The physiological mechanisms of excitation and adaptation have been examined in several photoreceptor cells of invertebrates. In two preparations, the lateral ocelli of *Balanus* (H. M. Brown, Hagiwara, Koike & Meech, 1970) and the ventral photoreceptor cells of *Limulus* (Millecchia & Mauro, 1969b), these studies employed a 'voltage-clamp' technique in which a single photoreceptor cell was impaled with two micropipettes. One of the pipettes measured the voltage between the inside of the cell and the extracellular bath (called the 'intracellular voltage') and the other passed the current necessary to hold fixed the measured voltage while the photoreceptor was illuminated. The voltage-clamp current was measured by a conventional current-to-voltage converter that held the bath at virtual ground. Current induced by illumination is then interpreted to be proportional to the light-induced change of membrane conductance, because neither membrane voltage nor the effective equilibrium voltage for ions that carry the membrane currents (Millecchia & Mauro, 1969b; Lisman & Brown, 1971) changes during the response to a brief stimulus.

There are two implicit assumptions in interpreting such 'point-clamp' experiments. First, during the light response, there must be uniformity of intracellular voltage across all parts of the cell over which light induces a change in conductance; i.e. the cell soma must be isopotential. If this assumption were not true then light would induce currents to flow between separate regions of the soma membrane. Secondly, the electrical resistance within the extracellular spaces in series with the light-activated parts of the cell membrane (the 'series resistance') must be small compared to the resistance of the light-activated membrane. If this second assumption were not true, then although the intracellular voltage recorded by the micropipette might remain fixed, the voltage across the cell membrane itself would not be fixed. If either assumption were false, then the recorded current would not necessarily be proportional to the light-induced conductance. These two assumptions have been made implicitly in many previous voltage-clamp studies on invertebrate photoreceptors (cf. Millecchia & Mauro, 1969b; Lisman & Brown, 1971, 1975a, b; Brown & Lisman, 1975; Coles & Brown, 1976; Fein & Charlton, 1977). It is known that the rhabdomere of *Limulus* ventral photoreceptors, comprised of microvilli of the plasma membrane, is found both on the surface of the soma of the photoreceptors and also along invaginations into the cell soma (Clark, Millecchia & Mauro, 1969; Stell & Ravitz, 1970). The extracellular space within these invaginations might offer a significant resistance in series with the membrane resistance of the rhabdomere, as has been found in the topologically similar photoreceptor cells of the leech (Fioravanti & Fuortes, 1972).

In this paper, we examine experimentally these two assumptions for the ventral photoreceptor cells of *Limulus polyphemus*. First, spatial uniformity of intracellular voltage has been tested by experiments in which three micro-electrodes were inserted into each cell. Secondly, the series resistance has been shown to be small by a technique employing measurements of the membrane voltage itself using a voltage-sensitive dye. The idea for using a voltage-sensitive dye to detect a series resistance is not novel; Davila, Cohen, Salzberg & Shrivastav (1974) have pointed out that in squid giant axons, using a voltage-clamp that is not compensated for series resistance, a dye signal can record a component of intracellular voltage that is proportional to the product of the series resistance and the membrane ionic current. However, when the series resistance was compensated in their voltage-clamp, Davila *et al.* (1974) found that the component of the dye signal proportional to the ionic current was eliminated.

METHODS

Ventral rudimentary eyes (Demoll, 1914; Clark *et al.* 1969) were dissected, pinned onto Sylgard 184 (Dow Corning, Midland, MI) and bathed in artificial sea water containing 425 mM-NaCl, 23 mM-MgCl$_2$, 25·5 mM-MgSO$_4$, 10 mM-KCl, 10 mM-CaCl$_2$ and 10 mM-Tris-HCl adjusted to pH 7·8. Before micro-electrode penetration, the tissue was bathed in Pronase (Calbiochem, LaJolla, Ca., grade B) 20 mg/ml. in artificial sea water for 1 min; the enzyme solution was removed by prolonged rinsing. Micropipettes were filled with 2 M-KCl and had resistances of 4–10 MΩ measured in artificial sea water. AgCl coated Ag wires were placed in the pipettes and were connected to M4 (WPI, New Haven, Conn.) preamplifiers to measure voltages. The circuit for the voltage-clamp was similar to that described by Millecchia & Mauro (1969b). A Bak ELSA 1 (Electronics for Life Sciences, Rockville, Md.) was used as the preamplifier for the micropipette that passed voltage-clamp current. The feed-back amplifier was a Tektronix (Beaverton, Or) 1A7A powered by a Tektronix 132 power supply; the gain was 6–20 × 10^3 and the high

frequency cut-off was $1-3 \times 10^3$ Hz. The bath was connected to virtual ground through a AgCl coated Ag wire inserted into a 2 mm diameter tube whose end was constricted to less than 0·5 mm, and whose lumen was filled with 2 M-KCl in 2% agar; this electrode had less than 1 kΩ resistance.

Micro-electrodes were placed onto a photoreceptor cell while the cell was observed with a 200 power compound microscope. The micro-electrodes were manipulated so that their axes were approximately 60° from the optical axis of the microscope. The cell membrane was penetrated by a micropipette by causing the preamplifier to oscillate (by overcompensating the input capacitance). After penetration, the electrode position was readjusted so that the recorded resting voltage and receptor potentials had maximum amplitudes. For experiments in which a cell was impaled by more than one micropipette, each pipette was manipulated separately. After each micropipette had been placed into the cell, it was advanced or withdrawn to achieve both maximum resting voltage and maximum receptor potential amplitude elicited by a bright light. Often the responsiveness of a cell was reduced after multiple impalements; such cells were discarded. We accepted only cells from which the two or three intracellular pipettes recorded receptor potentials that had identical shape and were equipotential to within 3 mV at their maximum (saturation) amplitude. Usually separate readjustment of the position of the pipettes was required to achieve these criteria for acceptable recordings. We think that these adjustments of pipette positions are required because the cell is mechanically distorted near the site of impalement by a micropipette.

Optical recording using a voltage-sensitive dye

The technique for the optical recording of changes of membrane voltage was modified from Salzberg, Grinvald, Cohen, Davila & Ross (1977). The measuring beam was produced by a 100 W tungsten iodide lamp (64625, Osram, Munich) powered by a current regulated power supply (model KS 18-15, Kepco, Flushing, N.Y.); the measuring beam was passed through a red glass filter (RT 830, Hoya Corp., Tokyo) and an interference filter with centre wave-length 760 nm (30 nm FWHM). The measuring beam had an irradiance of 7×10^{-6} W/cm² on the preparation. The stimulus beam was produced by a 100 W tungsten iodide lamp powered by a variable transformer (392533, Zeiss, Oberkochen); the stimulus beam was passed through a KG-1 glass filter (Schott, Duryea, Pa.), through an interference filter that blocked long and short wave-lengths (with cut-off wave-lengths at 625 and 365 nm) and through neutral density filters. An iris diaphragm in the stimulus beam was used to restrict the stimulus illumination to a region as small as 300 μm diameter on the preparation. The measuring and stimulus beams were combined by 40:40 mirror beamsplitter and were focused upward onto the preparation by a microscope condenser (N.A. 0·6). In order to eliminate small variations of path length through the pool that would otherwise be caused by waves at an air–water interface, a coverglass was fixed to the chamber immediately above the preparation to cover the pool; thus the light path of the measuring beam did not cross an air–water interface. Micropipettes were positioned to enter the pool at a shallow enough angle to pass under the edges of the coverglass. Light transmitted through the preparation was collected by a long working distance microscope objective (Leitz Wetzlar UM 32, N.A. 0·3), was passed through an interference filter with centre wave-length 750 nm (20 nm FWHM) and was focused onto a silicon photodiode (PV 100A; EG & G, Salem, Mass.) operated in the unbiased mode. The light reaching the photodiode was restricted to that transmitted through a single photoreceptor by two pairs of razor blades that defined a rectangular aperture in the back focal plane of the objective. The unattenuated stimulus beam evoked no measurable signal from the photodiode. The entire apparatus was mounted on a vibration isolation table (Newport Research Labs; Fountain Valley, Ca.).

Ventral photoreceptor cells were stained with a voltage-sensitive merocyanine-rhodanine dye (denotated WW-375, or dye XVII, by Ross, Salzberg, Cohen, Grinvald, Danila, Waggoner & Wang, 1977), at 0·5–1 mg/ml. in artificial sea water for 10 min. The dye solution was prepared immediately before staining the tissue. After staining, the ventral nerve was washed in artificial sea water for several minutes so that free dye was removed. The nerve noticeably lost stain during this wash. All solutions were equilibrated with air.

Although we were able to record dye signals for up to 4–5 hr after staining cells, the absorbance of dye bound to cells and the signal-to-noise ratio for dye signals decreased during an experiment. These decreases in dye signals were probably due in part to continued desorption of dye through-

out an experiment and in part due to bleaching of membrane-bound dye by the measuring beam. Therefore, we presented the measuring beam only for the time beginning immediately before and ending after the response to each stimulus flash. After the measuring beam was turned on, the amplified output of the photodiode was reset to approximately zero volts (using a model 755 digital sample and hold, Hybrid Systems, Burlington, Mass.). The voltage dependent change in optical transmission of the dye could then be observed at high gain. This procedure allowed the display of a change of 1 part in 10^4 in optical transmission. The dye signals were recorded
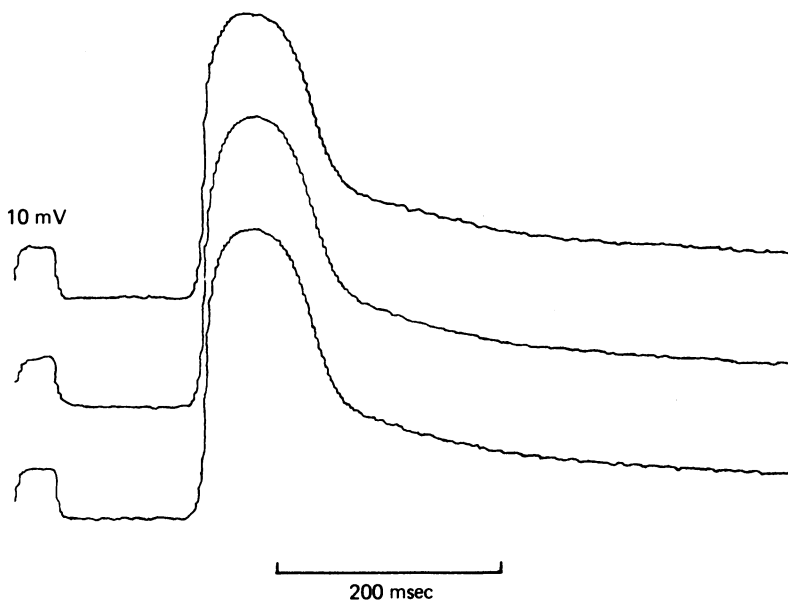

Fig. 1. Receptor potentials recorded simultaneously from three intracellular pipettes positioned as diagrammed in Fig. 2 *E*. At the beginning of each voltage trace there is a 10 mV calibration pulse.

simultaneously with electrode voltage and voltage-clamp current. Dye signals and electrical signals were averaged by a Nicolet 1072 signal averager (Madison, Wisc.); inputs to the signal averager passed through a low-pass filter with 1 kHz cut-off frequency. Because of the continuous desorption and bleaching of the membrane-bound dye, the base line of the optical signals steadily drifted upwards during the measurements (see Fig. 5 *A*); that is, there was a steady increase in optical transmission during each measurement. We subtracted a line that was matched in slope to the steady drift of the base line of each record in order to compare the optical signals with the electrical ones.

Anatomy

The cell somas of the photoreceptor cells in the ventral rudimentary eye of *Limulus* are mainly clustered at the distal end of the nerve with their axons running proximally to the brain. Also, some somas are randomly distributed singly and in clusters along the length of the ventral rudimentary eye (Demoll, 1914; Clark *et al.* 1969; cf. Fig. 1 in Holt & Brown, 1972). Several isolated photoreceptors usually can be found on each ventral rudimentary eye. The photo-receptors are oblong cells that are up to 150–200 μm long and 50–60 μm wide. The cells often have one or a few large lobes; the axon usually arises proximally from the cell body, but occasion-ally arises along a flank. All the recordings in this paper were made from cells clearly isolated from other photoreceptors to eliminate contributions to either voltage-clamp currents or optical signals arising from electrical coupling between adjacent photoreceptors.

<div align="center">RESULTS</div>

Spatial uniformity of the voltage-clamp

To examine the uniformity of intracellular voltage across the soma of photo-receptors, we impaled cells with three micropipettes. The pipettes were widely spaced on the cell soma with one pipette placed close to the origin of the axon. Two of the pipettes were used to measure intracellular voltage and the third used to pass voltage-clamp current. First, the receptor potentials elicited by a bright stimulus were recorded by each electrode (Figs. 1, 2 A, 3 A). If the receptor potentials could not be made to be identical at their maximum amplitude by repositioning the micropipettes, the cell was discarded. Then for each acceptable cell, first one, then the second, voltage electrode was clamped to resting voltage and the cell was stimulated by flashes bright enough to saturate the light-induced current (Fig. 4; cf. Brown & Coles, 1979, accompanying paper). The unclamped electrode measured any deviation from the clamping voltage (Figs. 2 B, C, 3 B, C). After each voltage-clamp trial, the voltage-clamp was disconnected and receptor potentials were again examined to verify that all the pipettes were still placed intracellularly and still met our criteria for acceptable recordings (Figs. 2 D, 3 D). As seen for two examples with different geometries for electrode placements (Figs. 2, 3), the unclamped electrode did not detect any marked deviation ($\leqslant$ 2 mV in each of five cells) from the clamping voltage, even for stimuli bright enough to saturate the light-induced current. That is, we have been unable to detect any marked spatial non-uniformity of intracellular voltage using our voltage-clamp procedure.

Voltage sensitive dye recordings

After bathing a *Limulus* ventral rudimentary eye in a solution of WW 375, some of the photoreceptor cells appear darkly stained. If impaled with a micropipette, these cells are found not to generate receptor potentials. Receptors that do respond to illumination appear pale blue, and usually have slightly darker bands (often annular bands) randomly positioned on the cell soma. These bands of staining probably correspond to bands of rhabdomere on the cell soma (Clark *et al.* 1969; Stell & Ravitz, 1970). Illumination of these stained receptors induces a receptor potential that can be recorded both electrically by a micropipette and as a change in optical transmission of the dye at 750 nm, ΔT (Figs. 5 and 7). The signal to noise ratio for a single response can be as large as 15.

Dye signals can also be recorded from the axons of cells whose somas are illumin-ated. These signals have slower rate of rise, longer time-to-peak, and smaller ampli-tude than the light-induced ΔT recorded from a soma. This finding is consistent with the electronic propagation of the receptor signal down the axons, as was discussed by Millecchia & Mauro (1969a) and has been demonstrated in *Balanus* photoreceptors by Shaw (1972) and Hudspeth, Poo & Stuart (1977). Two procedures were employed to ensure that dye signals from axons underlying a particular soma being studied did not contribute to the total optical signal. First, the stimulus was confined to a spot 300 μm in diameter centred on a chosen, isolated cell; therefore there would be little or no stimulation of cells whose axons might lie under the chosen cell. Secondly,
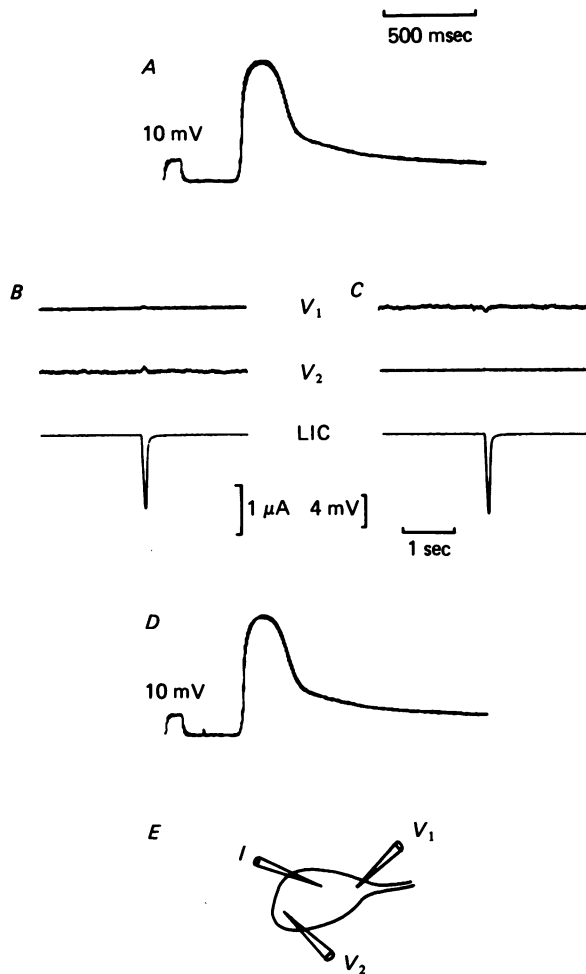
Fig. 2. Test for spatial uniformity voltage. The cell was impaled with three micro-pipettes. *A*, superimposed receptor potentials (shown separately in Fig. 1) recorded by each pipette initially before voltage clamping. At the beginning of each voltage trace there is a 10 mV calibration pulse. *B*, the voltage measured by pipette marked V_1 was held fixed by the voltage-clamp circuit. *C*, the voltage measured by pipette marked V_2 was held fixed by the voltage-clamp circuit. In neither case did the voltage recorded by the uncontrolled pipette deviate markedly from the voltage recorded by the controlled pipette. The light-induced currents are on traces labelled LIC. *D*, superimposed receptor potentials recorded by each pipette after voltage-clamping. *E*, diagram of the geometry of pipette placement. The stimulus irradiance was 6.5×10^{-7} W/cm^2; stimulus duration was 20 msec.
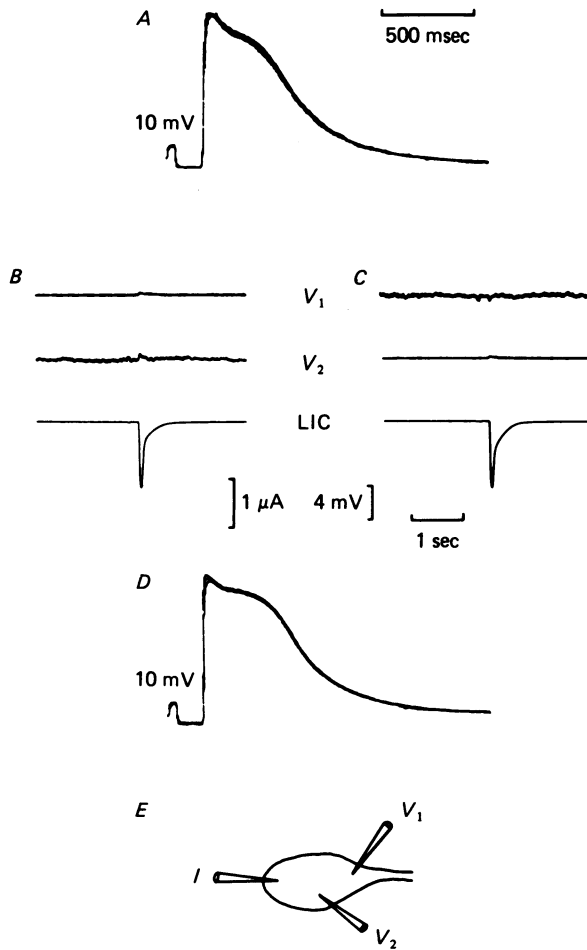
Fig. 3. Test for spatial uniformity of intracellular voltage. See Fig. 2 caption for description. The stimulus irradiance was 11.1×10^{-6} W/cm^2.

before recordings of ΔT were made from a cell soma, the aperture for the optical measurement was positioned distally, immediately adjacent to the soma, so that any contribution to an optical signal from other cell bodies located more distally could be detected. If such signals could be recorded (Fig. 5), the cell was not used for further analysis. Thus, with the aperture for the optical measurement restricted to the image of the soma, the optical signals were recorded from the single chosen cell (as in Figs. 6 and 7).
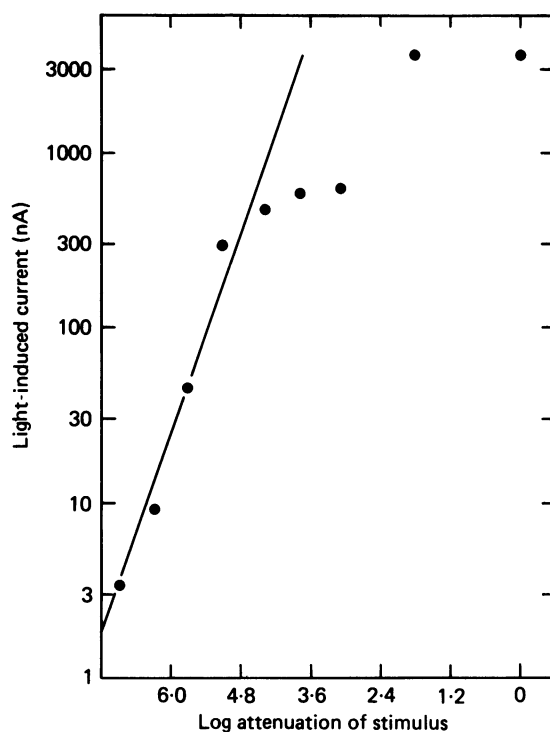

Fig. 4. Light-induced current as a function of stimulus irradiance. The cell was dark-adapted for 10 min; responses were recorded to successively brighter stimuli given 1/30 sec. As shown in Fig. 3, there was no spatial non-uniformity of intracellular voltage detected at even the brightest level of stimulus irradiance. Unattenuated irradiance: $11 \cdot 1 \times 10^{-6}$ W/cm².

To examine systematically the pharmacological effects of the dye, WW 375, on the light response of *Limulus* ventral photoreceptors, each of two cells was voltage-clamped and graphs of log peak light-induced current *versus* log stimulus irradiance were constructed both before and after staining with the dye. The maximum decrease in sensitivity of the cell after staining was 0·6 log unit and the peak currents induced by the brightest stimuli were decreased by a factor of 2.

The relationship between intracellular voltage and ΔT was examined in dark-adapted photoreceptors. This is seen for two cells in Fig. 6. Each cell was impaled with two micropipettes. For one cell (Fig. 6A) a current ramp was injected through one pipette while voltage was recorded by the second simultaneously with the optical
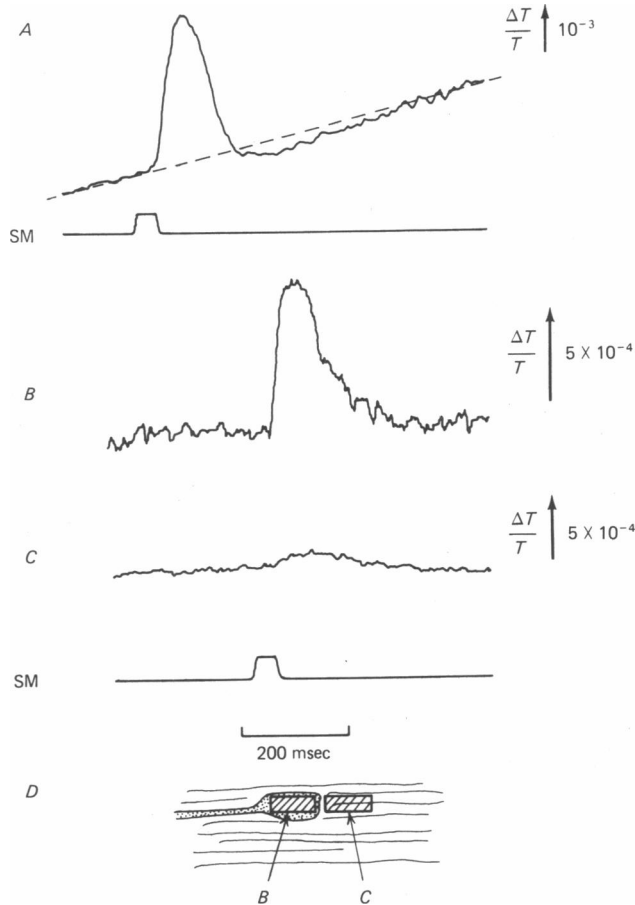
Fig. 5. Light-induced changes in optical transmission of a *Limulus* ventral photo-receptor stained with a voltage-sensitive dye (WW 375). The fractional changes in optical transmission are indicated by the arrows. *A*, the averaged dye signal (eight trials) recorded from the soma of a photoreceptor. The base line in this 'uncorrected' record slopes upward due to dye desorption and bleaching. The base lines for all other dye signals shown in this paper were 'corrected' by subtracting a line that was matched for each case to the steady upward drift of the base line (shown dashed). *B*, the dye signal recorded from another photoreceptor in a single sweep. The location of the aperture of the measuring beam was positioned over the soma as indicated in *D* by the arrow labelled *B*. *C*, the average dye signal (eight trials) recorded from the same cell as *B*. The aperture of the measuring beam was positioned immediately distal to the soma as indicated in *D* by the arrow labelled *C*. This signal was generated by axons arising from cells located more distally on the nerve. Stimulus irradiance: $1\cdot4 \times 10^{-8}$ W/cm^2. Stimulus monitor: SM. *D*, diagram of the placement of the aperture of the measuring beam for *B* and *C*.

transmission. In the other example, the cell was voltage-clamped using a ramp voltage as the command voltage for the feed-back amplifier (Fig. 6*B*). In both cases, the ΔT can be scaled linearly to be approximately congruent with the voltage.

These findings would indicate that ΔT is approximately linearly related to intracellular voltage over the physiological range of voltage. Therefore, ΔT would be linearly related to membrane voltage if the resistance in series with the membrane is

small compared to the resistance of the membrane in the dark. The membrane resistance in the dark, determined incrementally near resting voltage in *Limulus* ventral photoreceptors impaled with two micro-electrodes, is greater than 10^6 Ω (e.g. Millecchia & Mauro, 1969a; Brown & Coles, 1979, accompanying paper). The series resistance for cells in the dark is difficult to determine accurately by electrophysiological technique, because it is small (< 50 kΩ) compared to the membrane resistance
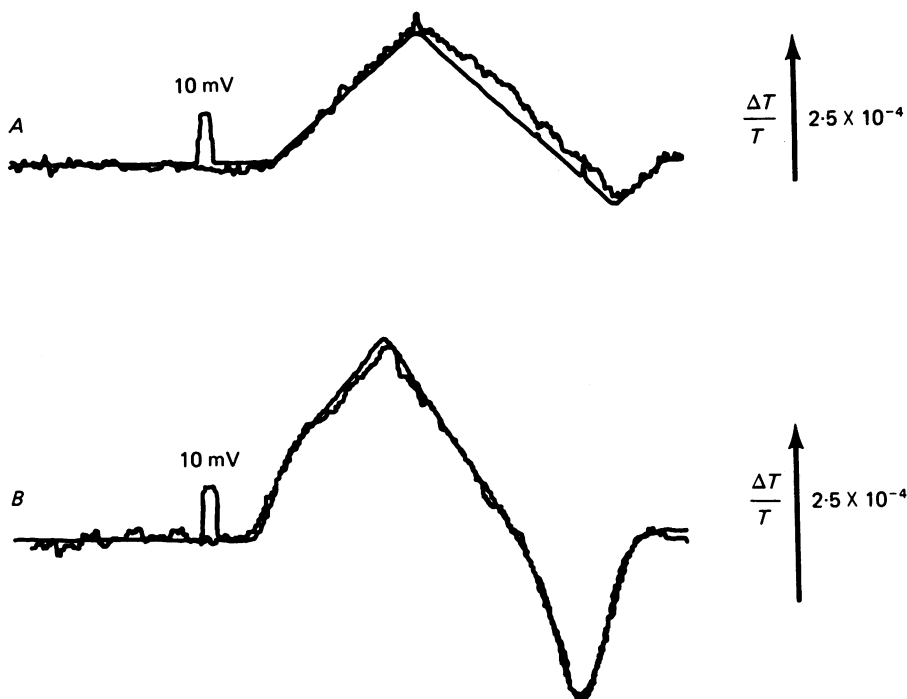

Fig. 6. Change in optical transmission as a function of intracellular voltage. Photoreceptor cells were impaled with two micropipettes. Optical transmission was recorded simultaneously with intracellular voltage. The percentage changes in optical transmission are indicated by the arrows. *A*, the cell was voltage-clamped; the command voltage for the feed-back amplifier was one period (20 sec duration) of a biased 'sawtooth' signal. The average ΔT (sixteen trials) is approximately linear with intracellular voltage. *B*, in another cell, a 'sawtooth' current (period = 20 sec) was injected through one micropipette and voltage recorded by the second micropipette. The average ΔT (eight trials) is approximately linear with intracellular voltage. Voltage calibration pulses recorded by the intracellular micropipettes were 10 mV.

(Brown & Coles, 1979, accompanying paper). Therefore, both an electrophysiological technique (Brown & Coles, 1979, accompanying paper) and an optical technique have been used to measure the series resistance during light responses, when membrane resistance is very much reduced.

Calculation of series resistance

   Because we apparently have good spatial uniformity of intracellular voltage using our voltage-clamp technique, there is no light-induced change in the voltage, ΔV_e,

between the voltage electrode inside the cell and the virtual ground. Therefore

$$\Delta V_e = 0 = \Delta V_m - \Delta i . R_s$$

That is, if the series resistance, R_s, is large compared to the membrane resistance during illumination, there will be a detectable voltage change across the membrane itself, ΔV_m, equal in size and opposite in sign to the Ohmic voltage dropped across the series resistance. Similarly, if we add a known resistance, R, in series with the unknown series resistance,

$$\Delta V_e = 0 = \Delta V_{m,R} - \Delta i_R . (R_s + R)$$

and

$$\Delta V_e = 0 = \Delta V_{m,o} - \Delta i_o . (R_s),$$

where the subscripts denote the value of the added resistance. We assume that the voltage-sensitive dye detects only changes in membrane voltage, and a change in optical transmission of the dye, ΔT, is directly proportional to a change in membrane voltage

$$k_o . \Delta V_{m,o} = \Delta T_o$$

and

$$k_R . \Delta V_{m,R} = \Delta T_R.$$

If we can arrange that the proportionality constants are the same with and without the added known resistor then

$$k_R = k_o = k$$

and the equations can be solved for R_s.

$$R_s = \frac{\Delta T_o . \Delta i_R . R}{(\Delta T_R . \Delta i_o - \Delta T_o . \Delta i_R)}.$$

Thus R_s can be calculated if the light-induced current and change in optical transmission of the voltage-sensitive dye can be measured simultaneously both with and without an added series resistance of known value.

Experimental determination of series resistance

With the cells voltage-clamped, we were unable to detect any change in ΔT elicited by a single stimulus. To improve the signal-to-noise ratio for the detection of ΔT, we averaged responses to many stimuli. The proportionality constant, k, relating ΔT to ΔV_m changes continuously during an experiment probably due to both desorption and bleaching of dye. Therefore, responses with and without the added series resistance were taken in alternating pairs so that the systematic change in the proportionality constant relating ΔV_m to ΔT did not distort the averages. From the records and the average peak amplitude of the light-induced currents that were simultaneously recorded (Fig. 7), we calculated that the series resistance, R_s, was $\leqslant 30$ kΩ. The values for five cells are given in Table 1.
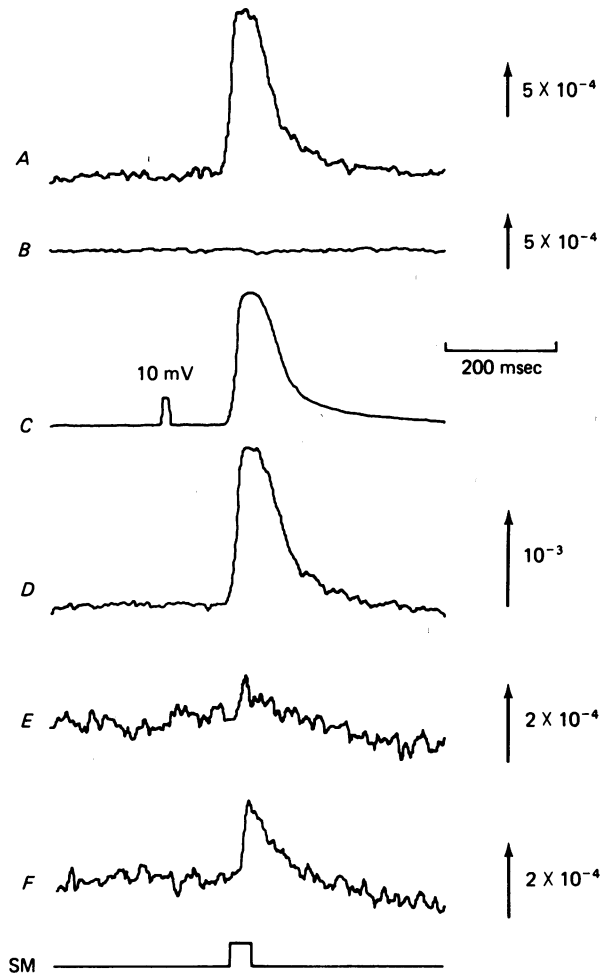
Fig. 7. Measurement of series resistance. Records are shown from cell no. 4 in Table 1. *A*, dye response elicited by a single stimulus. *B*, no average dye signal was detectable from axons arising from more distal photoreceptors. *C*, average receptor potential and, *D*, average dye signal recorded simultaneously (four trials). The cell was voltage-clamped using two intracellular micropipettes. The average dye signal (eight trials) was recorded simultaneously with membrane voltage and light-induced current (not shown). Alternating pairs of responses were recorded without and with an added series resistance. *E*, the average dye signal with no added series resistance. *F*, the average dye signal with 100 kΩ added series resistance. Stimulus irradiance: $1\cdot4 \times 10^{-8}$ W/cm². Stimulus monitor: SM.

TABLE 1.

(See text for definitions of symbols.)

Cell no.	Δi_o (nA)	Δi_R (nA)	$\dfrac{\Delta T_o}{T}$	$\dfrac{\Delta T_R}{T}$	R_s $(10^3\ \Omega)$	Unclamped case $\dfrac{\Delta T}{T} \cdot \dfrac{1}{\Delta V_m}$
1	65	55	$\leqslant 0\cdot27 \times 10^{-4}$	$1\cdot8 \times 10^{-4}$	$\leqslant 29$	0·026
2	55	49	$\leqslant 0\cdot44 \times 10^{-4}$	$3\cdot6 \times 10^{-4}$	$\leqslant 25$	0·023
3	34	30	$0\cdot27 \times 10^{-4}$	$1\cdot8 \times 10^{-4}$	15·5	0·020
4	38	36	$0\cdot80 \times 10^{-4}$	$3\cdot5 \times 10^{-4}$	26	0·066
5	37	33	$\leqslant 0\cdot05 \times 10^{-4}$	$0\cdot34 \times 10^{-4}$	$\leqslant 30$	0·014

<div align="center">DISCUSSION</div>

Spatial uniformity of membrane voltage

The membrane of the cell body of a ventral photoreceptor cell of *Limulus* is apparently equipotential when voltage-clamped using the procedures described in this paper. That is, we have been unable to detect significant spatial non-uniformities of intracellular voltage if all electrodes are adjusted to maximize the electrical responses of the cell. Moreover, spatial non-uniformities of intracellular voltage do not appear even when the cell is stimulated with lights bright enough to saturate the light-induced current, and when either a voltage-measuring or current-passing electrode is placed at the origin of the axon. These conditions should be the least favourable for spatial uniformity of intracellular voltage because the space constant of the soma membrane will be greatly reduced by the light-induced increase in membrane conductance whereas there is no evidence that there is a light-induced conductance change along the axon. Our findings do not exclude the possibility that some regions of the soma membrane of some cells are normally not equipotential; however if such non-uniformities occur, recordings from such cells would be discarded by our procedure.

It has long been recognized that voltage-clamping using two micropipettes cannot produce adequate spatial uniformity of voltage for all cell and electrode geometries (Cole, 1968). An extensive mathematical treatment of the spatial non-uniformity of membrane voltage for a spherical cell into which current is injected by a point source has been provided by Eisenberg & Engel (1970). They treat the case in which both the pipette that measures voltage and the pipette that passes current are placed just inside the cell membrane on radii with varying angular separation. This analysis demonstrates that the angular separation between current and voltage electrodes markedly influences the spatial uniformity of voltage if the separation becomes small. For a spherical cell 70 μm in radius, with $R_m = 10^3 \Omega$ cm^2, $R_i = 100 \Omega$ cm and a membrane time constant of 10^{-3} sec, Eisenberg & Engel (1970) calculate that for angular separations greater than 20° and times longer than 10^{-3} sec, the spatial non-uniformity will be less than 0·5 %. Inasmuch as the irregular geometry of the *Limulus* ventral photoreceptor can be represented as a sphere 70 μm or less in radius, the analysis predicts that 'point-clamping' with electrodes widely spaced ought to allow adequate spatial uniformity of membrane voltage. However, if the current and voltage electrodes are very closely spaced, as might be the case with 'double-barrel' electrodes or separate pipettes glued together, the theory predicts that significant deviations from spatial uniformity may occur. This theoretical consideration may account, in part, for differences in findings between workers who use a pair of very closely spaced pipettes (Srebro & Behbehani, 1974; Behbehani & Srebro, 1974) and those who have used the technique originally employed by Millecchia & Mauro (1969*b*) and described in this paper.

The relationship between peak current induced by brief stimuli and stimulus irradiance for a cell that showed no significant deviations in intracellular voltage is seen in Fig. 4. For this cell, this relationship shows several properties already noted in the literature. The slope of the curve increases from being approximately unity

for the least bright stimuli (Lisman & Brown, 1975a; Fein & Charlton, 1977). For stimuli approximately three orders of magnitude brighter than that necessary to elicit a single 'discrete event', the current begins to saturate (Lisman & Brown, 1975a; Brown & Coles, 1979, accompanying paper). For very bright stimuli, an additional increase in current and a second plateau of saturation can be observed when the cell is incompletely dark-adapted between stimuli (Brown & Blinks, 1974; Brown & Coles, 1979, accompanying paper). Because all these phenomena have been observed in a cell for which good spatial uniformity of intracellular voltage has been demonstrated, it is unlikely that these phenomena can be attributed to inadequate control of membrane voltage in the cell soma.

Voltage-sensitive dye

Changes of the optical absorption of the voltage-sensitive merocyanine-rhodanine dye (WW-375) can be used to measure changes in membrane voltage (ΔV_m) of *Limulus* ventral photoreceptors. As has been found in other membranes (Ross *et al.* 1977; Cohen & Salzberg, 1978) ΔT is directly proportional to ΔV_m over the physiological range of membrane voltages. However, staining the photoreceptor membrane with WW-375 does produce some changes in its physiology; we have recorded smaller values for peak light-induced currents from stained cells than from unstained ones. Moreover, stained cells do not seem to maintain the ability to generate large light-induced currents over as long a period as do unstained cells. These debilities of stained photoreceptors may arise from pharmacological action of the dye or may be due to photodynamic damage (Cohen & Salzberg, 1978).

Determination of series resistance

The values for series resistance of *Limulus* ventral photoreceptors found in the present experiments were approximately at the limit of detection for the technique employing voltage-sensitive dyes. That is, for many cells any signal attributable to the naturally occurring series resistance was not measurably greater than the noise. The noise in the optical measurements arises partly from vibration (probably due to movements of the tissue and the lamp filament) and is partly inherent in the quantal nature of the measuring light. For the latter, the signal-to-noise ratio increases roughly as the square root of the irradiance; however, the photoreceptor responds to, and is adapted by, the measuring beam. For this reason the measuring beam cannot be made arbitrarily bright; the cell would be desensitized by the measuring beam and the subsequent stimulus would induce a smaller current. Hence, the sensitivity for the detection of a series resistance would not necessarily be increased. In addition, a brighter measuring beam may produce both more bleaching of the membrane-bound dye and more photodynamic damage to the membrane. In sum, we consider the calculated numbers in Table 1 to be the upper limit for the series resistance; the smaller numbers ($R_s \simeq 15$–20 kΩ) are probably the more reliable. By comparison, Brown & Coles (1979, accompanying paper) have used an electrophysiological technique to determine the series resistance to be 7–24 kΩ.

From the calculated values of R_s and the measured values of light-induced currents, we can calculate the change of membrane voltage induced during the light response

while the voltage recorded by the intracellular pipette was held clamped. The values range from 0·5 to 1·9 mV. Another way to calculate the change in membrane voltage is from the relationship.

$$k_o . \Delta V_{m,o} = \Delta T_o.$$

To do this calculation, the value of k_o would have to be known for the time of the measurement of ΔT_o. We measured both $\Delta V_{m,o}$ and ΔT_o while the cell was not clamped at the beginning of each experiment, but did not determine k_o continually through the experiment. Therefore, the calculation will be in error due to the decrease in k_o as a function of time (due to dye desorption and bleaching). Nevertheless, for cells nos. 3 and 4 of Table 1 for which there was a measurable ΔT_o greater than the noise, $\Delta V_{m,o}$ was 1·4 and 1·2 mV respectively (compared with 0·5 and 1·0 mV from the Ohm's law calculation). It should be noted that the largest light-induced currents can exceed 1 μA (e.g. in Fig. 4) and for these large currents, a series resistance could cause the membrane voltage to change by more than 15 mV.

In conclusion, we have examined two assumptions commonly made for interpreting voltage-clamp experiments on invertebrate photoreceptors and found the assumptions to be justified for *Limulus* ventral photoreceptors impaled with widely spaced micropipettes. That is, the intracellular voltages measured by micropipettes widely spaced on the soma are equal; significant deviations of intracellular voltage are not found in voltage-clamped cells. Also, the series resistance is small enough so that light-induced currents of less than 100 nA principally reflect properties of the membrane conductances. These assumptions, although justified for *Limulus* ventral photoreceptors studied with the techniques described, cannot necessarily be generalized to other photoreceptors. A notable counter-example has already been described for leech photoreceptors by Fioravanti & Fuortes (1972).

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