Ionic Mechanism for the Generation of Horizontal Cell Potentials in Isolated Axolotl Retina

G. WALOGA and W. L. PAK

From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. Dr. Waloga's present address is the Department of Physiology, State University of New York, Stony Brook, New York 11794.

ABSTRACT The ionic mechanism of horizontal cell potentials was investigated in the isolated retina of the axolotl *Ambystoma mexicanum*. The membrane potentials of both receptors and horizontal cells were recorded intracellularly while the ionic composition of the medium flowing over the receptor side of the retina was changed. The membrane potential of the horizontal cell is highly dependent on the extracellular concentration of sodium. When the external ion concentration of either chloride or potassium was changed independently of the other, there were shifts in the membrane potential of the horizontal cell which could not be explained by changes in the equilibrium potential of these ions. If the external concentrations of both potassium and chloride ions were varied so that the product of their external concentrations did not change, the shift in the membrane potential of the horizontal cell was in the direction predicted by the Nernst equation. The results are consistent with the suggestion that in the dark the receptors release a synaptic transmitter which increases primarily the sodium conductance of the horizontal cell postsynaptic membrane.

INTRODUCTION

Horizontal cells were the first vertebrate retinal neurons to be investigated by intracellular recording techniques (Svaetichin, 1953). Although the operating characteristics of horizontal cells have been extensively investigated, the mechanisms for maintaining their membrane potential in the dark and generating their light responses are still not well understood. In this study, we have attempted to determine the ionic requirements for establishing membrane potentials of horizontal cells.

Anatomical studies suggest that the transmission of information between photoreceptors and second-order neurons is chemically mediated. Ribbon synapses, with synaptic vesicles, are found in all receptor terminals (De Robertis and Franchi, 1956; Missotten, 1965; Gray and Pease, 1971). It has been proposed (a) that there is a continuous release of a depolarizing synaptic transmitter from the presynaptic terminals of the photoreceptors to the postsynaptic membrane of horizontal cells in the dark (Trifonov, 1968; Byzov and Trifonov, 1968), and (b) that light causes hyperpolarization of the photoreceptors (Bortoff,

1964), thereby diminishing the amount of transmitter released. Thus, illumination would lead to a decrease in the amount of depolarizing transmitter impinging upon a horizontal cell, thereby creating the hyperpolarizing light response. Attempts have been made to influence the release of transmitter from photoreceptors by changing the ionic environment. Either an increased external magnesium ion concentration or a decreased external calcium ion concentration, or both, causes an interruption of excitatory transmitter release in many neural junctions (Del Castillo and Katz, 1956; Katz and Miledi, 1967; Colomo and Erulkar, 1970). If the release of chemical transmitter from retinal neurons behaves in a similar manner, one might expect the above manipulations of divalent cations to attenuate the release of transmitter from photoreceptors. In several preparations, such attempts have resulted in hyperpolarization of the horizontal cells and a decrease in the amplitude of the horizontal cell light responses (Dowling and Ripps, 1972, 1973; Cervetto and Piccolino, 1974; Trifonov et al., 1974; Kaneko and Shimazaki, 1975). However, in the isolated retina of the fish (Negishi and Sugawara, 1972) and marine toad (Pinto and Brown, personal communication), a decrease in external calcium ion concentration caused horizontal cells to depolarize and horizontal cell light responses to disappear.

Attempts to find the reversal voltage for the horizontal cell light response have resulted in contradictory data. In most cases, a reversal voltage within physiological bounds has not been found (MacNichol and Svaetichin, 1958; Gouras, 1960; Watanabe et al., 1960; Byzov, 1967; Negishi, 1968; Nelson, 1973). On the other hand, Trifonov et al. (1971) reported a reversal voltage at about 0 mV, and Werblin (1975) estimated reversal voltage at $+15$ to $+50$ mV. Resistance measurements during horizontal cell light responses have also produced contradictory results. During light responses, membrane resistance has been reported to increase (Tomita, 1965; Toyoda et al., 1969; Maksimova and Maksimov, 1971, for moderate light intensities), decrease (Maksimova and Maksimov, 1971, for near-saturating light intensities), or remain unchanged (Tasaki, 1960; Trifonov and Utina, 1966). Thus the details of mechanisms generating the horizontal cell light responses at present remain obscure.

Manipulating the ionic environment of a nerve cell while recording the membrane potential intracellularly has been a useful electrophysiological technique for investigating the ionic mechanisms of neural potentials. Recently, neurons in the vertebrate retina have been studied by this technique (Cervetto and MacNichol, 1972; Cervetto and Piccolino, 1974; Miller and Dacheux, 1973, 1976; Brown and Pinto, 1974). We investigated the ionic mechanism for potentials recorded intracellularly from horizontal cells in the isolated retina of the axolotl *Ambystoma mexicanum* (Waloga, 1975; Waloga and Pak, 1976). Since horizontal cells are second-order neurons, any changes induced by alterations of their ionic environment may be a composite of several changes. There could be a change in the receptor response, a change in the amount of synaptic transmitter release, or an effect on the horizontal cell membrane. Each of these changes might contribute to the observed change in horizontal cell activity. Ultimately, we will have to understand all these contributions to understand changes of horizontal cell activity. In order to understand part of the contribu-

tion resulting in changes of horizontal cell activity, we recorded intracellularly from both photoreceptors and horizontal cells while the ionic composition of the medium flowing over the receptor side of the retina was changed. A preliminary account of some of our results has already been presented (Waloga and Pak, 1976).

MATERIALS AND METHODS

Experimental Material

Axolotls *Ambystoma mexicanum* were dark adapted for at least 8 h. Under dim red light, an eye was removed and the retina was isolated in a pool of fresh, oxygenated control bathing solution. The retina was positioned receptor-side up and pinned in a chamber

TABLE I

COMPOSITION OF BATHING SOLUTIONS

Solution	NaCl	Choline-Cl	MgSO₄	KCl	CaCl₂	Na isethionate
Control solution	109	0	0	2.4	0.85	0
85% Na_0	92.65	16.35	0	2.4	0.85	0
65% Na_0	70.85	38.15	0	2.4	0.85	0
50% Na_0	54.5	54.5	0	2.4	0.85	0
26% Na_0	27.25	81.75	0	2.4	0.85	0
1% Na_0	0	109	0	2.4	0.85	0
20 mM Mg_0	109	0	20	2.4	0.85	0
0 Ca_0	109	0	0	2.4	0	0
300% K_0	109	0	0	7.2	0.85	0
0 K_0	109	0	0	0	0.85	0
68% Gl_0	72.7	0	0	2.4	0.85	36.3
36% Cl_0	36.3	0	0	2.4	0.85	72.7
4.5% Gl_0	0	0	0	2.4	0.85	109
89% Na_0	97	12	0	2.4	0.85	0
600% $[K_0]$ 16.7% $[Cl_0]$	1.9	0	0	14.4	0.85	95.1

All solutions contain 5 mM dextrose, 0.5 mM MgCl₂, 0.6 mM Na₂SO₄, 0.32 mM NaHCO₃, and 2.8 mM HEPES (N-2 hydroxyethylpiperazine N-2 ethane sulfonic acid) adjusted to pH 7.8 with NaOH. All tabulated values are mM concentrations.

the inside surface of which was coated with Sylgard (Dow Corning, Midland, Mich.). The chamber was similar to that used to investigate *Bufo marinus* rods (Brown and Pinto, 1974). An uninterrupted flow of oxygenated solution passed through the chamber at a rate of approximately 4 ml/min.

Solutions

We identify the concentration of an ion by a chemical symbol and a subscript i or o to indicate intracellular or extracellular, respectively. When we refer to the physiologically normal concentration of an ion, the chemical symbol is italicized. For example, the sodium concentration in a bathing solution which is 50% of the concentration in the control solution is denoted by 50% Na_0. The composition of each solution is shown in Table I. Oxygenated, glass-distilled water was used in all the solutions.

Light Stimulus

The test light beam originated from a xenon arc lamp, passed through a grating monochromator (Bausch & Lomb, Rochester, N. Y.), and was attenuated by Wratten

neutral density filters (Eastman Kodak Co., Rochester, N. Y.), and Barr and Stroud neutral density wedges (National Instrument Co., Rockville, Md.). The neutral density combination was calibrated at 430, 525, and 580 nm with a spectroradiometer (Gamma Scientific, San Diego, Calif., model 2020). The radiance of the attenuated light beam was 0.15 mW/cm² at 430, 0.14 mW/cm² at 525, and 0.14 mW/cm² at 580 nm. During the experiments, the beams were attenuated by 2–7 log units. The background light used in some experiments came from a tungsten-iodide lamp, passed through a 500-nm interference filter (Baird-Atomic, Bedford, Mass.) and neutral density wedges. Both the background and test lights diffusely illuminated the whole retina during most experiments. However, when needed, the apertures through which the beams passed could be changed in size and shape to allow rough receptive field measurements for identification of cell types.

Electrodes

Microelectrodes were filled with 4 M potassium acetate, and had resistances between 200 and 400 MΩ when measured in the control solution. A 3 M KCl-agar filled capillary was connected to a Ag-AgCl wire used as the reference electrode and placed downstream from the retina. When the bathing solutions were changed with both the recording and reference electrodes immersed, the difference in junction potentials was less than 2 mV, and was often undetectable.

Experimental Procedure

The type of cell penetrated by a microelectrode was identified by the waveshape of the light response, size of receptive field, and spectral sensitivity. To determine the last, a series of light stimuli which spanned about four log units of intensity was delivered at 430, 525, and 580 nm. These wavelengths were chosen to be close to the wavelength of maximum absorption (λ_{max}) of the different receptor pigments found in the axolotl retina: red rods, 515 nm; green rods, 430 nm; and cones, 575 nm (Liebman and Parkes, personal communication).

The effects of changing extracellular ion concentration were studied in both receptors and horizontal cells. Both the membrane potential in the dark and the light-induced responses were monitored before, during, and after each change of bathing solution. Only data from cells that showed recovery of membrane potentials after return to the control solution were retained. This procedure insured that the observed changes in potentials recorded with different bathing solutions were not due to changes in electrode position or irreversible damage to the preparation.

Resistance measurements were performed as described by Pinto and Pak (1974). A current clamp was used to force a sinusoidally modulated current of constant frequency (30 Hz) across the membrane via the intracellular microelectrode. Changes in membrane resistance caused changes in the transmembrane voltage induced by the current. A lock-in amplifier (see Smith et al., 1967) was used to measure changes in transmembrane voltage.

RESULTS

Light-Response Waveshapes

Although horizontal cells appeared to vary in their relative balance of rod and cone input (determined by spectral sensitivities), the waveshapes of the light responses measured in control bathing solutions had certain common characteristics. Horizontal cells had resting membrane potentials (dark potentials) of 15–

40 mV, inside negative. When the light was turned on, the membrane potential became more negative (Fig. 1 A). The light-induced hyperpolarization was maintained for the duration of the stimulus. The amplitude of the hyperpolarization increased with increasing light intensity over a 5-log unit range, until it saturated with very bright lights. When the light was turned off, the membrane began to return to the dark potential. After termination of the stimulus, responses induced by light of moderate to saturating intensities often developed a secondary hyperpolarization of a smaller amplitude that decayed slowly to the dark-potential level (Fig. 1 A). In the case of a prolonged stimulus (longer than 0.5 s), a rapid, transient depolarization overshooting the dark-potential level
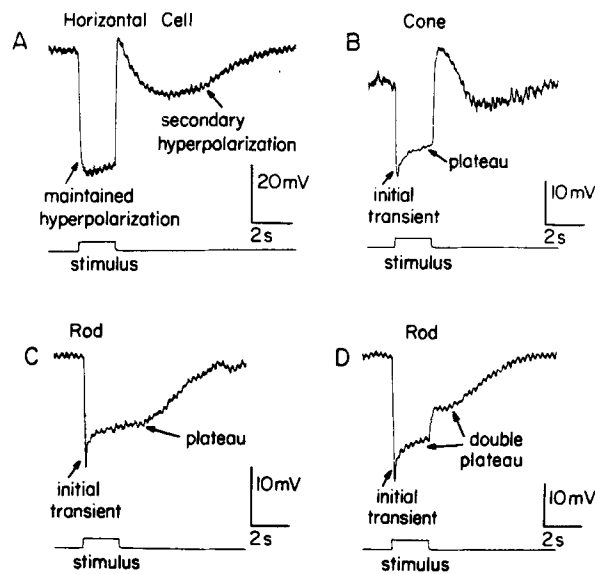
FIGURE 1. Waveshapes of horizontal cell light responses and receptor potentials. (A) Light response of a horizontal cell. (B) Cone receptor potential. (C) Receptor potential of the rod. (D) Rod receptor potential with a "double plateau." All cells are shown responding to a 2-s, 525-nm stimulus.

could be seen immediately after the termination of the stimulus, before the secondary hyperpolarization appeared (Fig. 1 A).

When the light-induced responses of horizontal cells were studied as functions of stimulus wavelength and intensity, the amplitude of the maintained hyperpolarization in some cells was found to be slightly wavelength dependent. Some cells gave the largest response (measured as peak millivolts per incident quanta) to 580-nm light at moderate and high intensities. We will refer to this type of horizontal cell as the 580 nm-sensitive cell. The spectral sensitivity is shown next to a typical light response at moderate light intensity (Fig. 2 A). When the light was turned off, the 580 nm-sensitive cell rapidly depolarized towards the dark-potential level. At very dim light intensities at which cone responses could not be detected, these horizontal cells became most sensitive to 525 nm light (not shown). Other cells were most responsive to 525 nm light at all intensities up to saturation. We will refer to this type of horizontal cell as the 525 nm-

sensitive cell. The spectral sensitivity is shown next to a typical light response at moderate light intensity (Fig. 2 B). When the light was turned off, the 525 nm-sensitive cells depolarized towards the dark-potential level much more slowly than the 580 nm-sensitive cells. The 525 nm-sensitive cells probably are driven primarily by the rods, since they are most responsive to wavelengths of light close to λ_{max} of the red rod pigment.

Cones responded to light stimuli with a transient, initial hyperpolarization
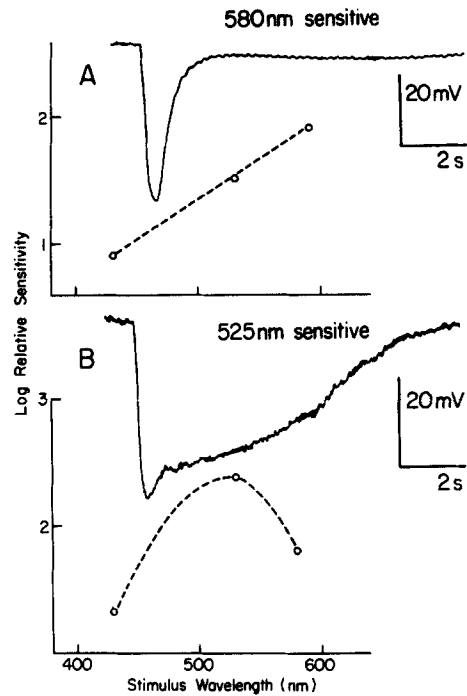
FIGURE 2. Spectral sensitivity of horizontal cells. Thresholds were determined at three different wavelengths by measuring the relative number of quanta needed to produce a 25-mV criterion response at each wavelength. Sensitivity equals 1/threshold. (A) Spectral sensitivity of a 580 nm-sensitive horizontal cell. (B) Spectral sen 525 nm-sensitive horizontal cell. Next to each spectral sensitivity curve is an example of light responses of 580 nm- or 525 nm-sensitive horizontal cells. Light stimulus is 200 ms at 525 nm.

that decayed to a less hyperpolarized plateau level. The plateau was maintained for the duration of the stimulus (Fig. 1 B). Both the latency and time to reach peak amplitude were shorter for cone responses than for horizontal cell responses. The amplitude of the cone light response increased with increasing light intensity until it saturated with very bright lights. When the light was turned off, the membrane potential rapidly returned to the dark level. If either the light stimulus approached a saturating intensity or the duration was increased, a transient depolarization followed by a hyperpolarization appeared at the termination of the stimulus (Fig. 1 B).

Illumination also elicited a hyperpolarization of rod membranes. The latency

and time to reach peak amplitude were similar for rods and cones. If the light intensity was brighter than that needed to produce a rod response of about half its saturated amplitude, the rod light response began to develop an initial transient that decayed to a maintained plateau hyperpolarization (Fig. 1 C). Both the initial transient and the plateau were graded with light intensity, although the transient saturated at an intensity about one log unit brighter than the saturation intensity for the plateau. For stimuli of low to moderate intensities, the plateau lasted about the same duration as the stimulus. However, at higher intensities, the plateau outlasted the stimulus and slowly returned to the dark potential. Some rods displayed double plateaus. In these rods, a bright stimulus evoked a plateau which was maintained for the duration of the stimulus. When the light was turned off, the receptor potential decayed to a less hyperpolarized second plateau and then slowly returned to the dark level (Fig. 1 D). The double plateau was observed in cells with relatively large responses (greater than 25 mV). Not enough data were collected to determine more precisely the conditions under which double plateaus could be reliably observed.

Contribution of Na_o to the Membrane Potential

An increase in input resistance of 5-10 $M\Omega$. accompanied the response of the horizontal cells to light, although a pronounced resistance increase was not observed in some of the horizontal cells tested. If a light-regulated sodium conductance is important in maintaining the membrane potential, substituting supposedly impermeant choline for sodium in the bathing solution should hyperpolarize the membrane in the dark and diminish the light response of the horizontal cell. When half of the Na_o was replaced by choline, the input resistance of both receptors and horizontal cells increased, the dark membrane potentials became hyperpolarized, and the light responses diminished (Fig. 3). The effects of reducing Na_o upon the light responses and dark potentials are plotted in Fig. 4 A and B, respectively.

For all test solutions, except the 26% Na_o (29 mM Na_o) solution, data were obtained from either 14 rods and 3 cones, or 13 horizontal cells. The data measured in the 26% Na_0 solution were obtained with either two rods and one cone, or two horizontal cells. For each cell, the amplitudes of the light responses obtained in the test solutions were normalized with respect to response amplitude for that cell measured in the control bathing solution. The amount of depolarization in the dark due to the presence of external sodium ions in the test solution was measured from the maximum hyperpolarization level obtained in the 1% Na_o solution. The amount of depolarization so obtained at each value of Na_o was normalized with respect to the amount of depolarization obtained in the same cell when bathed in the control solution, i.e.,

$$v_m = \frac{V_m(Na_o) - V_m(1\% Na_o)}{V_m(100\% Na_o) - V_m(1\% Na_o)},$$

where $V_m(Na_o)$ = membrane potential in the test solution, $V_m(100\% \ Na_o)$ = membrane potential in the control bathing solution, $V_m(1\% Na_o)$ = membrane potential in 1% Na_o solution. The dark potentials and the light-evoked responses

(receptor potentials) of receptor cells displayed nearly the same dependence upon Na_0 (Fig. 4). Likewise, the horizontal cell dark potential and its light responses displayed very similar dependences on Na_0 (Fig. 4). If the horizontal cell were not dependent upon Na_0 for maintenance of its membrane potential or for the generation of the light-evoked response, and if the synaptic transmission from receptors to horizontal cells were approximately linear, one would expect the amplitude of light response (and dark membrane potential) vs. Na_0 curve to be very similar for both horizontal cells and receptors. This was not the case. The amplitude of the light responses (and dark potential) of horizontal cells diminished much more with decreasing Na_0 than the receptor potentials
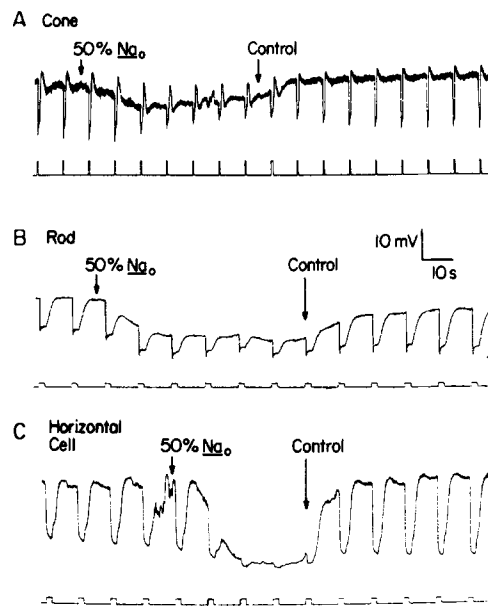
FIGURE 3.   The effect of 50% Na_0 solution upon receptor and horizontal cell potentials. The 50% Na_0 solution hyperpolarizes the membranes and decreases the receptor potentials in both (A) cones and (B) rods. (C) 50% Na_0 solution hyperpolarizes the membrane of the horizontal cell much more than the receptor membranes, and almost eliminates the light responses.

(and dark membrane potential) of the rods and cones (Fig. 4 A, B).

The difference in activity of receptors and horizontal cells in solutions having decreased Na_0 was examined further in the following way. We compared the reduction of response magnitude induced by background light with that induced by decreased Na_0 (Fig. 5). In the isolated retina, it was much easier to record from rods than cones. For this reason, this part of the investigation was limited to 525 nm-sensitive horizontal cells. After exchanging the control solution with a bathing solution containing slightly less than normal Na_0 (85% Na_0), the hyperpolarization of the rod membrane potential in the dark and the diminution of the receptor potentials were measured (Fig. 5 B). Then, in the control solution, the intensity of background light necessary to produce equivalent amounts of both steady hyperpolarization and reduction in
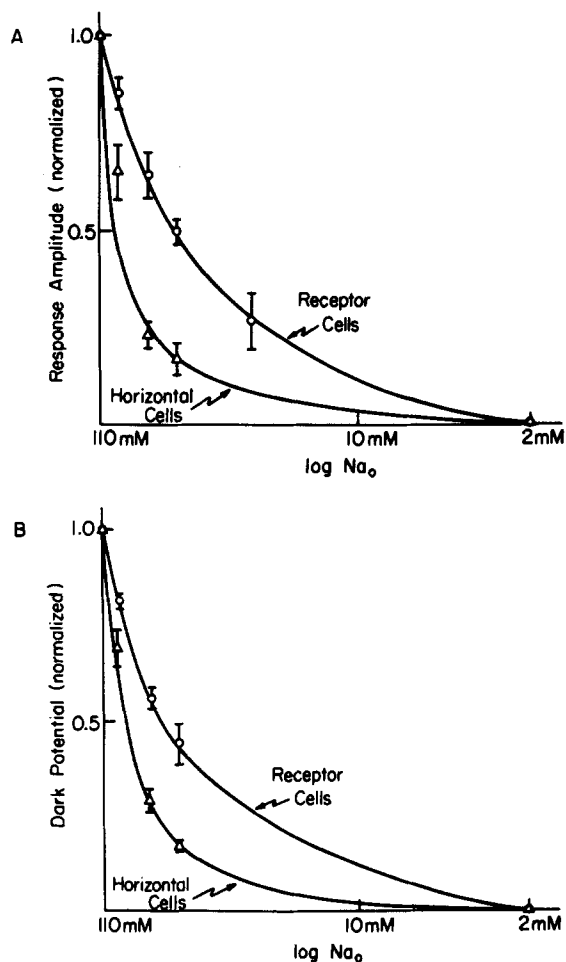
FIGURE 4. (A) The effect of decreasing Na_0 on the light responses of receptors and horizontal cells. The horizontal cell responses display much stronger dependence on Na_0 than the receptor potentials. Data are from cells which recovered completely upon return to the control solution after being bathed in several different Na_0 solutions. All points, except the 26% Na_0 (29 mM Na_0) were based on data obtained from either 14 rods and 3 cones, or 13 horizontal cells. The 26% Na_0 points were obtained with either two rods and one cone, or two horizontal cells. For each cell, the amplitudes of the light responses were normalized with respect to the response amplitude for the same cell in the control bathing solution. (B) Effect of decreasing Na_0 on the dark membrane potentials of receptors and horizontal cells. The membrane potential of the horizontal cell displays much stronger dependence on Na_0 than the membrane potential of the receptor cell. For each cell, the dark membrane potentials at various Na_0s were measured with respect to that obtained in 1% Na_0—taken to be very close to the maximum hyperpolarization obtainable in that cell. The "amplitude" of the membrane potential so obtained at each Na_0 (the membrane potential at Na_0 minus that at 1% Na_0) was then normalized with respect to the "amplitude" in control bathing solution for the same cell. All points were based on data obtained from either 14 rods and 3 cones, or 13 horizontal cells which recovered completely upon return to the control solution after being bathed in several different Na_0 solutions.

77

the receptor potentials (Fig. 5 C) was determined. The background light of this intensity will be referred to as equivalent-background light. Thus, the effect upon the rod membrane potential level was similar for both the 85% Na_0 solution and the equivalent-background light (Fig. 5 B, C). The effect of equivalent-background light was then compared to the effect produced by the 85% Na_0 solution upon the 525 nm-sensitive horizontal cells. The hyperpolarization of the membrane in the dark and the reduction of the light response amplitude were much more pronounced in the 85% Na_0 solution (Fig. 5 E) than in the presence of the equivalent background light (Fig. 5 F). The horizontal cells used in this experiment were most sensitive to 525-nm light when the dark potential was at its normal value as well as when hyperpolarized by the 85% Na_0 solution (Fig. 6); therefore, they were probably driven primarily by rods under both conditions.

Divalent Cations: Ca_0 and Mg_0

It has been found in most, if not all, chemical synapses studied that Ca^{++} is needed for transmitter release and that Mg^{++} antagonizes the effect of Ca^{++}. We increased Mg_0 or decreased Ca_0 to see if these divalent cations modulate the process of transmitter release in the retina also. If the receptor to horizontal cell synapse behaves similarly to other chemical synapses, these changes would be expected to diminish transmitter release. When we added 20 mM $MgSO_4$ to the control bathing solution, the dark membrane potential of the receptors and the receptor potential remained unchanged (Fig. 7 A, B). The horizontal cells, however, rapidly hyperpolarized when the additional 20 mM Mg_0 was present (Fig. 7 C). As the membrane of the horizontal cell began to hyperpolarize, the amplitude of the light response began to decrease and a slight, transient depolarization began to appear at stimulus offset (Fig. 7 C).

When Ca_0 was decreased, the membrane of the rod initially depolarized and the amplitude of the receptor potential increased (Fig. 8 A). Brown and Pinto (personal communication) found, however, that when the *Bufo marinus* retina was bathed in low Ca_0 for more than 5 min, the receptor potentials decreased in amplitude. The horizontal cell, on the other hand, depolarized and the light responses disappeared in low Ca_0 (Fig. 8 B). Upon return to the control solution, the horizontal cell membrane became slightly more hyperpolarized than it was before the low-Ca_0 solution was introduced. The light responses of horizontal cells were smaller and did not decay as rapidly; both the amplitude and the time course of decay took several minutes in the control solution to recover to their normal values.

Changes of K_0

In many nerve cells, the membrane potential is very dependent upon K_0. The axolotl retina was bathed in solutions containing either increased or decreased K_0. High K_0 (300% K_0) caused rods to depolarize slightly and the initial transient component of the receptor potential to disappear (Fig. 9 A and C). If the 300% K_0 solution bathed the retina for longer than 1 min, the recovery of the normal potentials in the control solution was very poor. Cones behaved in a manner similar to rods when the retina was bathed in 300% K_0 solution. The

dark membrane potential of cones was unchanged but the initial transient hyperpolarization was decreased in amplitude (Fig. 10). When the horizontal cells were bathed in 300% K_o, the membrane potentials drifted and became noisy (and the cells often hyperpolarized), and the light responses decreased in amplitude (Fig. 11 C, D). After return to the control solution, the membrane potentials took several minutes to recover if they had been in the 300% K_o solution longer than 1 min.
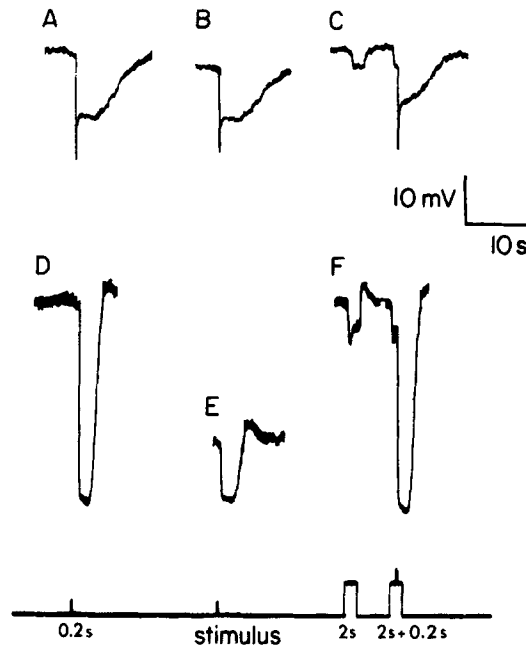

FIGURE 5. Reduction of response magnitude induced by either decreased Na_0 or equivalent background light. (A) Rod responds to a 0.2-s test stimulus in the control solution. (B) In 85% Na_0 solution, the rod is slightly hyperpolarized in the dark and the response to the test stimulus is diminished. (C) In control solution, the intensity of a 2-s background light (equivalent background) was determined which would produce equivalent amounts of both steady hyperpolarization and reduction in the receptor potentials. (D) The horizontal cell responds to a 0.2-s test stimulus in the control solution. (E) In the 85% Na_0 solution, the hyperpolarization of the membrane and the diminution of the response to the test stimulus are much greater than (F) those produced by the equivalent-background light in the control solution.

When the retina was bathed in a low-K_0 solution, there was no noticeable change in the dark membrane potential of rods or cones (Figs. 9 B and 10 B). Upon presentation of a light stimulus, the initial transient of receptor potentials was found to be slightly larger in the 0% K_o solution than in the control solution (Fig. 9 A, B). In both rods and cones, the plateaus remained at the same amplitude, but they began to return to the dark level sooner in 0% K_o solution than in the control solution (Figs. 9 A and 10 A). All receptor potentials recovered within a few seconds after the control solution was reintroduced. In
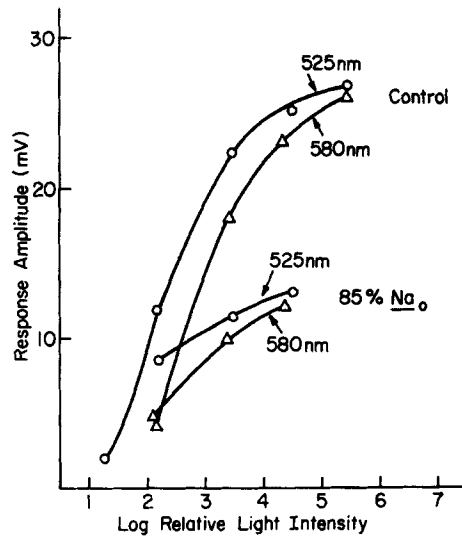
FIGURE 6. Comparison of stimulus-response curves for a 525 nm-sensitive horizontal cell in control solution, and in 85% Na_0 solution. The amplitude of the light responses is greatly diminished in the 85% Na_0 solution. However, the cell is still more sensitive to the 525 nm stimulus.
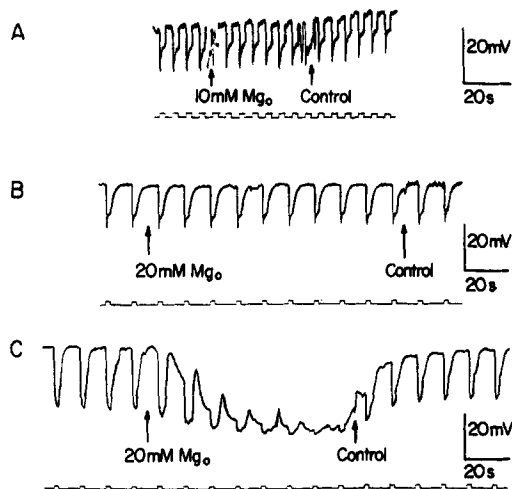

FIGURE 7. Receptor potentials and horizontal cell light responses in solutions containing high Mg_0. $MgSO_4$ added to the control solution does not noticeably change either (A) the cone receptor potentials or (B) the rod receptor potentials. (C), 20 mM Mg_0 added to the control solution hyperpolarizes the horizontal cell membrane and eliminates its light response.

horizontal cells, the 0% K_0 solution usually depolarized the membrane very slightly in the dark, and the waveshape of the light response was altered by the elimination or reduction in amplitude of any secondary hyperpolarization (Fig. 11 A, B).

Decreased Cl_o

With replacement of up to 95.5% of the Cl_o by either isethionate or methane sulfonate, the rods and cones showed neither noticeable change in dark potential nor change in receptor potentials (Fig. 12). However, horizontal cells were greatly affected by low Cl_0 solutions (Fig. 13). The membrane of horizontal cells hyperpolarized in low Cl_o solutions, and the amplitude of the light response progressively decreased with decreasing Cl_o in the bathing solution (Fig. 14). In addition, a transient depolarization appeared in the light response of horizontal cells when the light was turned off (Fig. 13 A). If the retina remained in the 4.5% Cl_o solution for over 2 min, the light responses were
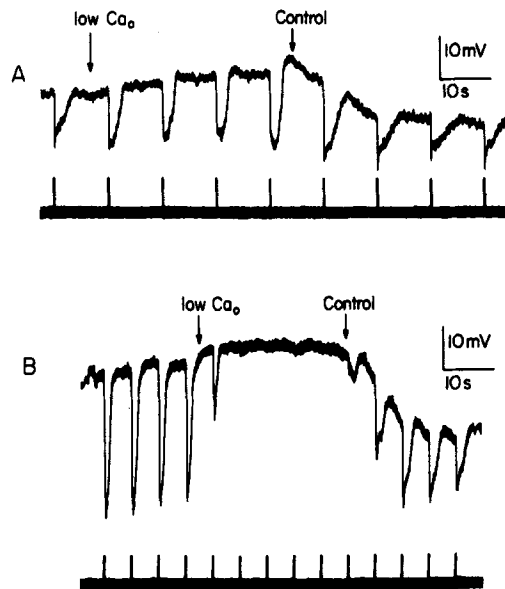

FIGURE 8. Receptor potentials and horizontal cell light responses in solutions with low Ca_0. (A) In 0% Ca_0 solution the rod depolarizes, the receptor potentials increase in amplitude, and the initial transient is lost. (B) In 0% Ca_0 solution, the horizontal cell depolarizes and the light responses are eliminated.

eliminated in most horizontal cells. Afterwards, recovery in the control solution took at least 5 min and was often incomplete.

Effect of Keeping the Product $[K_o][Cl_o]$ Constant

The changes in membrane potential of horizontal cells during alterations of K_0 and Cl_0 described above appear to be anomalous for biological membranes. That is, the changes in the dark membrane potential are of polarity opposite to that predicted by the Nernst equation. This behavior might be due in part to osmotic expansion or shrinkage of cell volume. Single muscle fibers, which have a high permeability to potassium and chloride ions, exhibit osmotic changes of cell volume unless the product $[K_0][Cl_0]$ is kept constant (Hodgkin and Horowicz, 1959; Boyle and Conway, 1941). It is possible that the nonsynaptic membrane of the horizontal cell is also highly permeable to potassium and chloride
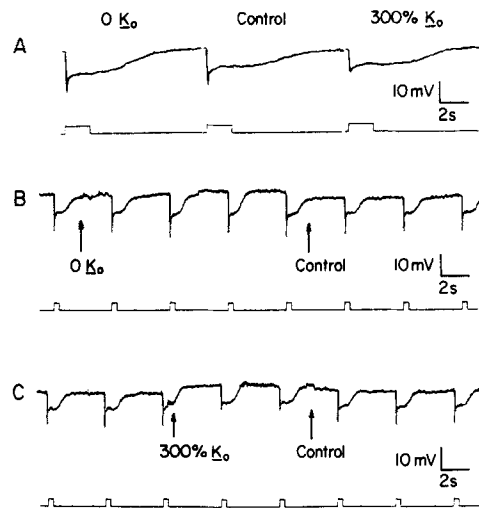
FIGURE 9. Receptor potentials of rods in low and high K_0. (A) Waveshapes of receptor potentials in 0% K_0, control, and 300% K_0 solutions. (B) In 0% K_0 solution, there is not much change in the membrane potential. (C) In 300% K_0 solution, the membrane potential depolarizes slightly.
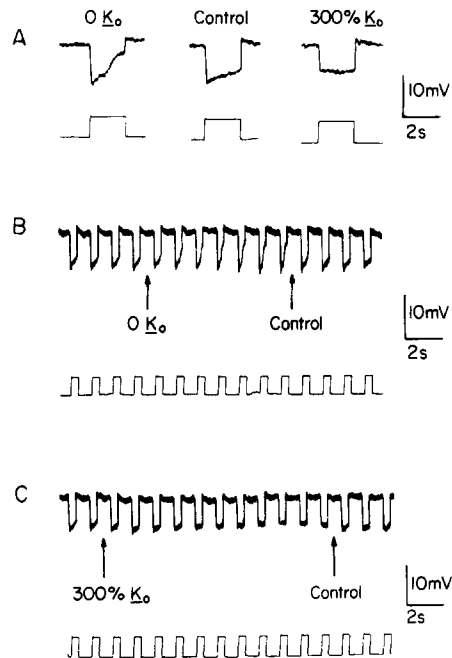

FIGURE 10. Receptor potentials of cones in high and low K_0 solutions. (A) Waveshapes of receptor potentials in 0% K_0, control, and 300% K_0 solutions. There is not much change in the dark membrane potential in either (B) the 0% K_0 solution or (C) the 300% K_0 solution.

ions and behaves similarly to the muscle fiber membrane. In an attempt to reduce osmotic changes of cell volume, we used test solutions having constant $[K_0][Cl_0]$ products.

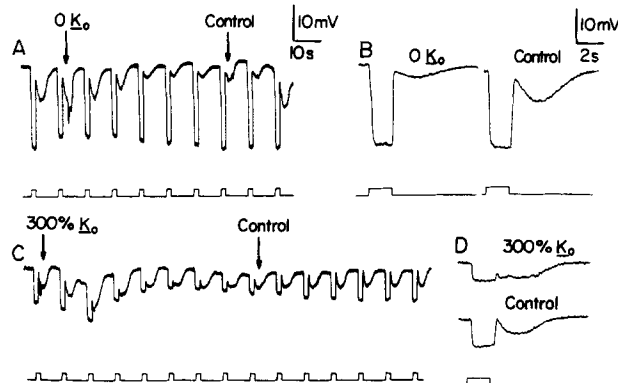When rods were bathed in a solution having 600% $[K_0]$ 16.7% $[Cl_0]$, the

FIGURE 11. Horizontal cell light responses in low and high K_0 solutions. (A) In 0% K_0 solution, the dark potential depolarizes very slightly, and (B) the off hyperpolarization decreases in amplitude. (C) 300% K_0 solution the membrane potential drifts, and (D) the light response diminishes in amplitude.
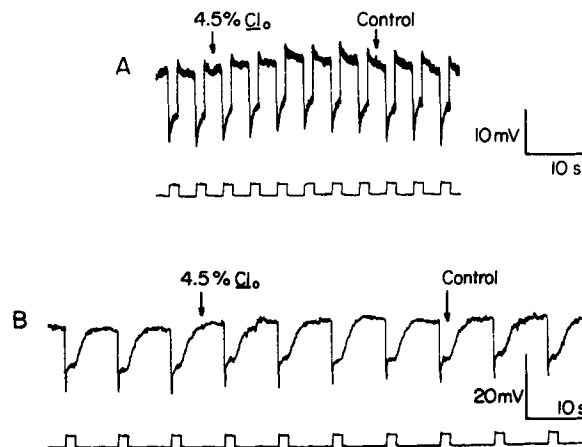

FIGURE 12. The effect of 4.5% Cl_0 solution upon receptor potentials. The 4.5% Cl_0 solution does not noticeably change the dark membrane potential or the receptor potentials of either (A) cones or (B) rods.

membrane became depolarized (Fig. 15 A) and the dark membrane conductance increased (not illustrated). There were also changes in the receptor potential of the rods. The initial transient was eliminated and the plateau remained hyperpolarized for a few seconds longer than it did in the control solution. The rods recovered their normal membrane potentials and conductance when the control solution was reintroduced into the chamber. When horizontal cells were bathed in the same solution, the membrane depolarized (Fig. 15 B)

without a noticeable change in the membrane conductance (not shown). Thus, the effect of a 600% $[K_0]$ 16.7% $[Cl_0]$ solution on the horizontal cell dark potential was opposite in polarity to that of either low-Cl_0 or high-K_0 solution. The light responses had smaller amplitude and longer latency to peak, and decayed more slowly to the dark level in the 600% $[K_0]$ 16.7% $[Cl_0]$ solution than in the control solution (Fig. 15 B).

DISCUSSION

Possible Ionic Mechanisms

Horizontal cells in the vertebrate retina respond to light stimuli by hyperpolarizing for the duration of the stimulus. Recent investigations have yielded
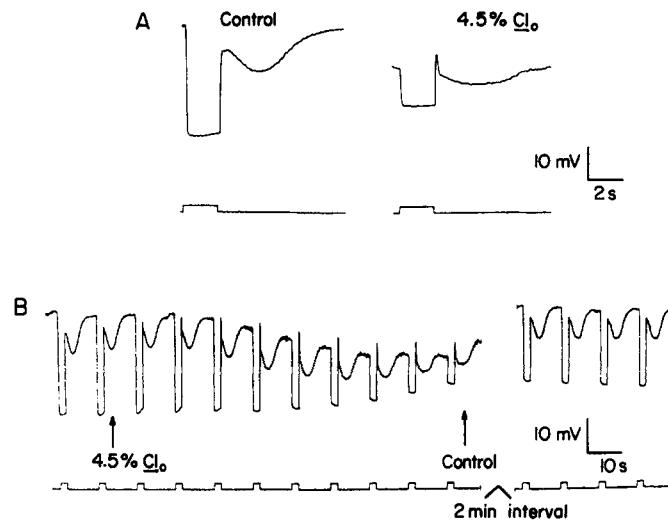

FIGURE 13. The effect of 4.5% Cl_0 solution upon horizontal cell light responses. In the 4.5% Cl_0 solution, (A) the light response decreases in amplitude, the waveshape changes, and (B) the cell becomes hyperpolarized. The break in the base line indicates a 2-min interval of recovery in the control solution.

results which tend to favor the following hypothesis for generation of light responses in horizontal cells (see Trifonov, 1968; Dowling and Ripps, 1973). In the dark, a transmitter continually released from the receptor terminals depolarizes the horizontal cell by increasing the permeability of the postsynaptic membrane for certain ion(s) whose equilibrium potential(s) is more positive than the membrane potential in the absence of the transmitter release. Light decreases the release of the transmitter and thus hyperpolarizes the horizontal cells.

The above hypothesis thus suggests that the light response of the horizontal cell is associated with a conductance decrease in the postsynaptic membrane of the horizontal cell. This light-induced decrease in conductance has been difficult to observe reliably, because the nonsynaptic membrane of the horizontal cell increases in conductance when hyperpolarized, partially obscuring the

conductance decrease in the postsynaptic membrane (Trifonov et al., 1971, 1974; Werblin, 1975). In the axolotl, the horizontal cell membranes show either a decrease or no change in conductance during the light response in the absence of extrinsic current. This finding suggests that, in axolotl horizontal cells, the conductance change in the subsynaptic membrane tends to dominate over that in the nonsynaptic membrane. Thus, in the dark, the conductance of the postsynaptic membrane is higher for an ion(s) whose equilibrium potential is more positive than those of other permeable ions. If the conductance of the ion(s) decreased during the light stimulus, the membrane would hyperpolarize to a more negative value.

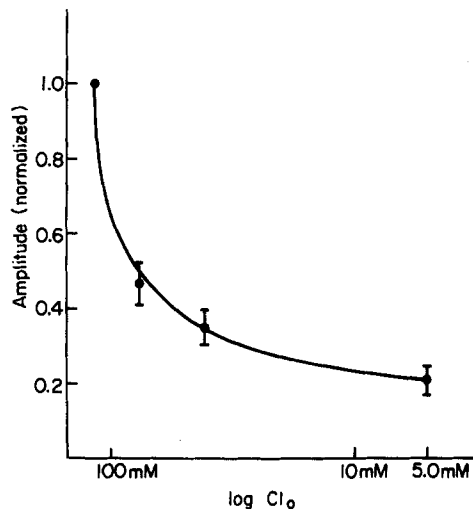In an effort to determine the ion(s) involved in the above process, we varied

FIGURE 14. The effect of decreasing Cl_0 upon the amplitude of horizontal cell light responses. The amplitude of the light response decreases with decreasing Cl_0 in the bathing solution. Data are from six horizontal cells. The amplitudes of the light responses for each cell were normalized with respect to the response amplitude for the same cell in the control bathing solution.

the Na_0. When the Na_0 was decreased, the horizontal cell membrane hyperpolarized, the membrane conductance decreased and the light-response amplitude diminished. Although the membrane potential of the receptors was also dependent upon Na_0, the effect upon horizontal cells was much more pronounced (Figs. 3 and 4). There are at least two possible explanations for the observed difference in the dependence upon Na_0 of the receptor and horizontal cell membranes. The difference might be attributable to either (a) nonlinearities in synaptic transmission (i.e., the amount of transmitter release is not linearly related to the membrane potential of the receptors), or (b) a sodium conductance playing an important role in maintaining the membrane potential of the horizontal cell. If the difference between receptor and horizontal cell dependences upon Na_0 were caused by nonlinearities in synaptic transmission, the receptor cell hyperpolarizations due to the 85% Na_0 solution and the equivalent-

background light should have the same effect upon the horizontal cell membrane potential. However, the 85% Na_0 solution hyperpolarized the horizontal cell and diminished the light response much more than the equivalent-background light (Fig. 5). Thus it does not appear likely that the greater dependence of horizontal cells upon Na_0 is due to nonlinearities in synaptic transmission between receptor and horizontal cells. Therefore, we conclude that (a) Na_0 appears to be important in maintaining the horizontal cell membrane potential in the dark, and (b) a light-decreased sodium conductance appears to participate in generating the light response. It has recently been suggested that sodium is also important in maintaining the membrane potential of carp horizontal cells (Kaneko and Shimazaki, 1975).
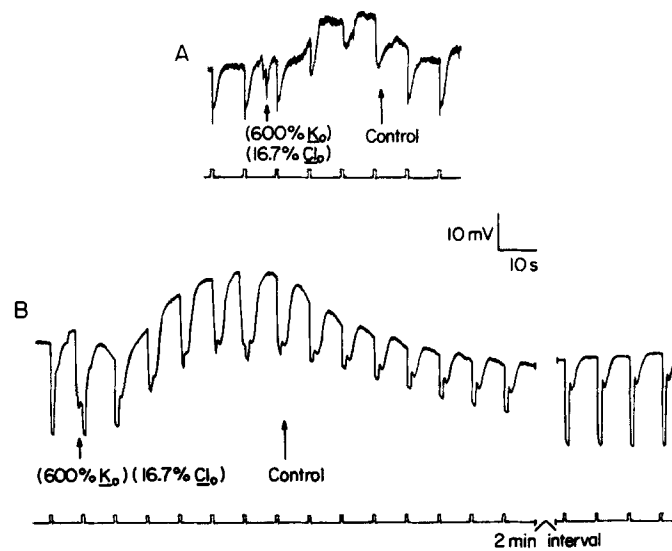

FIGURE 15.   The effect of keeping the product $[K_0]$ $[Cl_0]$ constant. In the 600% $[K_0]$ 16.7% $[Cl_0]$ solution, both (A) the rod and (B) the horizontal cell depolarize. The break in the base line is a 2-min recovery interval in control solution. Both solutions contained 89% Na_0.

In the above experiment, the equivalent-background intensity was determined by recording from rods rather than cones. For the above conclusions to be valid, the horizontal cells under study must have received their input primarily from rods at all levels of hyperpolarization used in the experiment. In the normal state, the cell was found to be most sensitive to the 525-nm stimulus (Fig. 6), suggesting its predominant rod input. Even in the 85% Na_0 solution, the cell still responded better to 525-nm stimuli than to 580-nm stimuli (Fig. 6). The results suggest that the rods predominate in providing input to the cell even at the most extreme level of hyperpolarization used in the experiment.

A possible alternative explanation of the above experiment might be that sodium is required for receptor-transmitter binding in the postsynaptic membrane and not necessarily involved in the ionic current through the postsynaptic

membrane. In the case of opiate receptors, Snyder and Matthysse (1975) have postulated that the presence of sodium renders the receptor into a form with a high affinity for opiate antagonists, while in the absence of sodium the same receptors exist in a form with a high affinity for agonists. Thus, the binding of antagonists to the receptors requires the presence of sodium. It would be difficult to distinguish between the mechanism we postulated for horizontal cells and one similar to the opiate system on the basis of our experiment. However, Snyder and Matthysse's opiate system appears sufficiently different from horizontal cells (e.g., antagonists rather than agonists require sodium for binding). Moreover, in the absence of comparable information on sodium dependence of the two systems, a further comparison does not seem worthwhile. In any case, the most likely interpretation of our results is that sodium is important in maintaining horizontal cell membrane voltage.

Divalent Cations

In many synaptic junctions, an increase in Mg_0 and a decrease in Ca_0 have been shown to cause a reduction of excitatory synaptic transmitter release (del Castillo and Katz, 1956; Katz and Miledi, 1967; Colomo and Eruklar, 1970). In axolotl retina, when 20 mM $MgSO_4$ was added to the bathing solution, the membrane potential of the rods appeared unchanged, but the horizontal cells became hyperpolarized and lost their light responses (Fig. 7). These findings are consistent with the hypothesis that high Mg_0 reduces the amount of depolarizing transmitter release, or may even block it. Similar results have been observed in the horizontal cells of other vertebrates (Dowling and Ripps, 1972, in skate; Cervetto and Piccolino, 1974, in turtle; Trifonov et al., 1974, in fish).

In the isolated axolotl retina, the waveform of the rod receptor potential appeared unaffected by the application of magnesium ions. Since in high Mg_0 horizontal cells lose their light responses, this result suggests that there is apparently no feedback from horizontal cells to rods in the isolated axolotl retina. Feedback inhibition from horizontal cells to cones has been indicated in eyecup preparations of turtle (Baylor et al., 1971) and gecko (Pinto and Pak, 1974). The few experiments that have been done on axolotl cones suggest that the addition of 10 mM $MgSO_4$ has no effect upon the cone receptor potential either.

One might also expect that decreased Ca_0 would cause a decrease in the release of transmitter from receptors and thus cause a hyperpolarization of the horizontal cell and a diminution of the light response. However, in the isolated axolotl retina, we found that markedly reduced Ca_0 (0% Ca_0 solution) depolarized the horizontal cell and eliminated the light response (Fig. 8). Similar results were obtained in the isolated retina of the marine toad (Brown and Pinto, personal communication) and isolated retina of the carp (Negishi and Sugawara, 1972). On the other hand, in the perfused turtle eyecup, the horizontal cells hyperpolarized and light responses diminished (Cervetto and Piccolino, 1974). The depolarization observed in 0% Ca_0 might be due to a direct effect of low Ca_0 on the horizontal cell membrane, in addition to any effects on transmitter release. For example, "destabilization" of horizontal cell membrane might occur in the absence of sufficient Ca_0 (Shanes, 1958), with a

consequent increase in permeability to sodium ions. This possibility has not been tested by measuring the membrane conductance or decreasing Na_o while in the low Ca_o solution. Such a destabilizing effect, if present, might predominate over any hyperpolarization that would arise from the reduction of depolarizing transmitter release caused by low Ca_o.

Manipulating K_o and Cl_o

Horizontal cell membranes hyperpolarized and the light response diminished when Cl_o was decreased (Fig. 13). Similar results were also shown in the rabbit retina (R. F. Miller, personal communication). *Necturus* horizontal cells, on the other hand, briefly depolarized and the amplitude of the light response increased first when exposed to low Cl_o. The horizontal cells then hyperpolarized, and the light response decreased in amplitude. A "chloride hypothesis" has been proposed to explain the results in *Necturus*. It suggests that an increased chloride conductance exists in the dark and that light reduces this conductance (Miller and Dacheux, 1976). In our experiments with axolotl, however, we have never observed either the initial depolarization of the cell membrane or the increase in light response, when the horizontal cell was exposed to low Cl_o. Thus, our results in the axolotl are not consistent with the "chloride hypothesis." In addition, the above changes in membrane potential of axolotl horizontal cells cannot be explained by simple changes in the equilibrium potential for chloride ions. Although the actual value of the chloride equilibrium potential is not known, one can nevertheless predict the direction of shift in equilibrium potential due to alterations in Cl_o. The observed shifts in membrane potential are opposite in polarity to those expected from the above considerations.

Increasing K_o usually slightly hyperpolarized the membrane of the axolotl horizontal cell and made the membrane potential drift, whereas decreasing K_o usually very slightly depolarized the membrane (Fig. 11). These results are anomalous in that they cannot be explained in terms of shifts in potassium equilibrium potential due to altered K_o. No satisfactory explanation can be offered for these results at the present time. In fact, it is even possible that the observed effects of altered K_o on the membrane potential might not closely reflect the changes in potassium equilibrium potential. For example, it might be argued that there is a potassium-induced transmitter release from retinal neurons which causes the seemingly anomalous shifts in horizontal cell membrane potential when K_o is changed.

In addition to hyperpolarization of the membrane, the light responses of horizontal cells diminished in amplitude in high K_o. There are at least two possible explanations for this effect. (a) The decrease in light response might reflect the small decrease in amplitude of receptor potentials. (b) Alternatively, if the nonsynaptic membrane is substantially more permeable to potassium ions than the subsynaptic membrane and the sodium mechanism for horizontal cell potentials holds, then high-K_o solutions might diminish the light responses without showing as big a change in the dark potential. The reason is that under the above conditions, the light stimulus would change the Na permeability in the subsynaptic membrane but not the K permeability in the nonsynaptic

membrane. Thus, the horizontal cell dark potential would depend on both the Na and K equilibrium potentials, while the light response would depend mainly on the K equilibrium potential. Therefore, the light response in high K_o is expected to be smaller than in low K_o. In support of the high K permeability in the nonsynaptic membrane, Kaneko and Shimazaki (1975) have shown that fish horizontal cells are very dependent upon K_o when synaptic transmission has been partially blocked.

It is rather unlikely that a simple relationship exists between changes in K_o, Cl_o, and osmotic volume, since the subsynaptic membrane of the horizontal cell is permeable to sodium ions. Nevertheless, if the nonsynaptic membrane of the horizontal cell were sufficiently permeable to K and Cl, we felt that it might be possible to reduce osmotic changes in cell volume by keeping the product of K_o and Cl_o constant. In such solutions, the rods depolarized in the dark, and the changes in waveshape of the rod receptor potential were similar to those observed in high K_o with normal Cl_o (Fig. 15 A). However, the horizontal cells depolarized in 600% $[K_0]$ 16.7% $[Cl_0]$ solution (Fig. 15 B), in contrast to the hyperpolarization observed in either 300% K_o or low Cl_o solutions. The membrane conductance, however, did not change. Although the observed depolarization is consistent with what would be expected from the Nernst equation, there are still too many variables that cannot be readily controlled to offer one simple explanation for the depolarization.

It is possible that some of the changes in low Cl_o (i.e. the solutions in which only Cl_o is varied) are the result of extreme shrinkage of parts of the horizontal cell. A decrease in cell volume would make the Na_i larger, and the sodium equilibrium potential less positive, thereby hyperpolarizing the cell. This idea has not been tested quantitatively, e.g., by making the horizontal cell shrink while maintaining normal values of K_o, Cl_o, and Na_o. The 30 mV hyperpolarization observed in a few horizontal cells in response to decreased Cl_o would require a volume decrease to about half its normal size. This seems unlikely to occur. When 20 mM glucose was added to the control solution (which might tend to shrink the cell), no change was observed in either receptor potentials or light responses of horizontal cells.

SUMMARY

The investigations of the ionic mechanism of the horizontal cell described in this study yielded results which are consistent with the following proposal. In the dark, the presynaptic terminals of the receptors release a transmitter, and the transmitter increases primarily the sodium conductance of the horizontal cell postsynaptic membrane. The membrane potential of the horizontal cell is dependent upon the ratio of sodium conductance to the conductance of each of the other permeable ions. The sodium equilibrium potential is more positive than the equilibrium potentials of the other ions likely to participate in maintaining the membrane potential. Therefore, the membrane is depolarized as long as the ratio of sodium conductance to conductances of other permeable ions remains increased. In the light, the amount of transmitter released by the receptors decreases; therefore the sodium conductance decreases, and the membrane potential becomes more negative.

REFERENCES

BAYLOR, D. A., M. G. F. FUORTES, and P. M. O'BRYAN. 1971. Receptive fields of cones
in the retina of the turtle. *J. Physiol. (Lond.).* **214**:265-294.

BORTOFF, A. 1964. Localization of slow potential responses in the *Necturus* retina. *Vision
Res.* **4**:627-635.

BOYLE, P. J., and E. J. CONWAY. 1941. Potassium accumulation in muscle and associated
changes. *J. Physiol. (Lond.).* **100**:1-63.

BROWN, J. E., and L. H. PINTO. 1974. Ionic mechanism for the photoreceptor potential
of the retina of *Bufo marinus. J. Physiol. (Lond.).* **236**:575-591.

BYZOV, A. L. 1967. Horizontal cells of the retina as regulators of synaptic transmission.
Neurosci. Transl. **3**:268-276.

BYZOV, A. L., and JU. A. TRIFONOV. 1968. The response to electric stimulation of
horizontal cells in the carp retina. *Vision Res.* **8**:817-822.

CERVETTO, L., and E. F. MACNICHOL, JR. 1972. Inactivation of horizontal cells in turtle
retina by glutamate and aspartate. *Science (Wash. D. C.).* **178**:765-768.

CERVETTO, L., and M. PICCOLINO. 1974. Synaptic transmission between photoreceptors
and horizontal cells in turtle retina. *Science (Wash. D. C.).* **183**:417-419.

COLOMO, F., and S. D. ERULKAR. 1970. The actions of calcium and magnesium on
synapses of spinal neurons in the frog. *Fed. Am. Soc. Exp. Biol. Proc.* **29**:391.

DEL CASTILLO, J., and B. KATZ. 1956. Biophysical aspects of neuromuscular transmission.
Prog. Biophys. Biophys. Chem. **6**:121-170.

DEROBERTIS, E., and C. M. FRANCHI. 1956. Electron microscope observations on
synaptic vesicles in synapses of the retinal rods and cones. *J. Biophys. Biochem. Cytol.*
2:307-318.

DOWLING, J. E., and H. RIPPS. 1972. Effects of Mg^{2+} on skate horizontal cells: evidence
for release of transmitter from receptors in dark. *Biol. Bull. (Woods Hole).* **143**:458-
459.

DOWLING, J. E., and H. RIPPS. 1973. Effect of Mg^{2+} on horizontal cell activity in the
skate retina. *Nature (Lond.).* **242**:101-103.

GOURAS, P. 1960. Graded potentials of the bream retina. *J. Physiol. (Lond.).* **152**:487-505.

GRAY, E. G., and H. L. PEASE. 1971. On understanding the organization of the retinal
synapses. *Brain Res.* **35**:1-15.

HODGKIN, A. L., and P. HOROWICZ. 1959. The influence of potassium and chloride ions
on the membrane potential of single muscle fibers. *J. Physiol. (Lond.).* **148**:127-160.

KANEKO, A., and A. SHIMAZAKI. 1975. Effects of external ions on the synaptic transmis-
sion from photoreceptors to horizontal cells in the carp retina. *J. Physiol. (Lond.).*
252:509-522.

KATZ, B., and R. MILEDI. 1967. A study of synaptic transmission in the absence of nerve
impulses. *J. Physiol. (Lond.).* **192**:407-436.

MACNICHOL, E. F., JR., and G. SVAETICHIN. 1958. Electric responses from the isolated
retinas of fishes. *Am. J. Ophthalmol.* **46**:26-46.

MAKSIMOVA, E. M., and V. V. MAKSIMOV. 1971. Changes in direct current input resistance in horizontal cells of fish retina during excitation. *Neirofiziologiya.* **3**:210–216.

MILLER, R. F., and R. F. DACHEUX. 1973. Information processing in the retina: importance of chloride ions. *Science (Wash. D. C.).* **181**:266–268.

MILLER, R. F., and R. F. DACHEUX. 1976. Synaptic organization and ionic basis of on and off channels in mudpuppy retina. I. Intracellular analysis of chloride sensitive electrogenic properties of receptors, horizontal cells, bipolar cells, and amacrine cells. *J. Gen. Physiol.* **67**:639–660.

MISSOTTEN, L. 1965. The synapse in the human retina. *In* The Structure of the Eye. II. Symposium, Wiesbaden. J. Rohen, editor. Schattauer, Stuttgart. 17–28.

NEGISHI, K. 1968. Excitation spread along horizontal and amacrine cell layers. *Nature (Lond.).* **218**:39–40.

NEGISHI, K., and K. SUGAWARA. 1972. Effects of various gases and drugs on the horizontal cell membrane potential in the isolated carp retina. *J. Physiol. Soc. Jpn.* **34**:546.

NELSON, R. 1973. A comparison of the electrical properties of neurons in *Necturus* retina. *J. Neurophysiol.* **34**:519–535.

PINTO, L. H., and W. L. PAK. 1974. Light-induced changes in photoreceptor membrane resistance and potential in gecko retinas. I. Preparations treated to reduce lateral interactions. *J. Gen. Physiol.* **64**:26–48.

SHANES, A. M. 1958. Electro-chemical aspects of physiological and pharmacological action in excitable cells. *Pharmacol. Rev.* **10**:59–164.

SMITH, T. G., R. B. WUERKER, and K. FRANK. 1967. Membrane impedance changes during synaptic transmission in cat spinal motoneurons. *J. Neurophysiol.* **30**:1072–1096.

SNYDER, S. H., and S. MATTHYSSE. 1975. Opiate receptor mechanisms. *Neurosci. Res. Prog. Bull.* **13**.

SVAETICHIN, G. 1953. The cone action potential. *Acta Physiol. Scand.* **29**(Suppl. 106):565–599.

TASAKI, K. 1960. Some observations on the retinal potentials of the fish. *Arch. Ital. Biol.* **98**:81–91.

TOMITA, T. 1965. Mechanism subserving color coding. *Cold Spring Harbor Symp. Quant. Biol.* **30**:559–566.

TOYODA, J., H. NASAKI, and T. TOMITA. 1969. Light-induced resistance changes in single photoreceptors of *Necturus* and *Gekko*. *Vision Res.* **9**:453–463.

TRIFONOV, JU. A. 1968. Study of synaptic transmission between photoreceptors and horizontal cells by means of electric stimulation of the retina. *Biofizika.* **13**:809–814.

TRIFONOV, JU. A., A. L. BYZOV, and L. M. CHAILAHIAN. 1974. Electrical properties of subsynaptic and nonsynaptic membranes of horizontal cells in fish retina. *Vision Res.* **14**:229–241.

TRIFONOV, JU. A., L. M. CHAILAHIAN, and A. L. BYZOV. 1971. Investigations of the nature of electrical responses of horizontal cells of the fish retina. *Neirofiziologiya.* **3**:89–98.

TRIFONOV, JU. A., and I. A. UTINA. 1966. Investigation into the mechanism of action of current on the L-type retinal cells. *Biofizika.* **11**:646–654.

WALOGA, G. 1975. Ionic mechanism for the generation of S-potentials: Horizontal cells in isolated axolotl retina. Ph.D. Thesis. Purdue University, West Lafayette, Ind.

WALOGA, G., and W. L. PAK. 1976. Horizontal cell potentials: Dependence on external

sodium ion concentration. *Science* (*Wash. D. C.*). **191:**964–966.

WATANABE, K., T. TOSAKA, and T. YOKOTA. 1960. Effects of extrinsic electric current on the cyprinid fish EIRG (S-potentials). *Jpn. J. Physiol.* **10:**132–141.

WERBLIN, F. S. 1975. Anomalous rectification in horizontal cells. *J. Physiol.* (*Lond.*). **244:**639–657.