J. Physiol. (1975), **252**, *pp.* 509–522
With 9 *text-figures*
Printed in Great Britain

EFFECTS OF EXTERNAL IONS ON THE SYNAPTIC TRANSMISSION FROM PHOTORECEPTORS TO HORIZONTAL CELLS IN THE CARP RETINA

BY A. KANEKO AND H. SHIMAZAKI

From the Department of Physiology, Keio University School of Medicine, Shinanomachi, Shinjuku-ku, Tokyo, Japan

(*Received 22 April 1975*)

SUMMARY

1. Intracellular recordings were made from cones and horizontal cells of the isolated carp retina and the mechanisms of synaptic transmission from cones to horizontal cells were studied by changing the ionic composition of the external medium.

2. Cones were depolarized and their light responses enhanced in low-Ca high-Mg medium. In the same medium, in which chemical transmission is supposed to be blocked, horizontal cells were hyperpolarized and their light responses disappeared.

3. When the synaptic input was removed, the membrane potential of horizontal cells agreed well with E_K.

4. In Na-free medium both cones and horizontal cells were hyperpolarized and response disappeared. On reapplication of normal Ringer, horizontal cells showed a transient membrane potential reversal (inside positive), indicating that the horizontal cell has a high Na permeability under the influence of the endogenous transmitter.

5. Application of La produced little change in cones, while it strongly depolarized horizontal cells. The depolarization is produced probably by a large amount of transmitter released from the receptor terminals by the action of La. Depolarization persisted in normal Ringer even after the removal of La, but a high membrane potential was restored in Na-free Ringer.

6. These observations support a hypothesis that the transmitter from cones is continuously released in the dark and the release stops when the receptors are illuminated. The transmitter depolarizes horizontal cells probably by increasing Na permeability.

INTRODUCTION

It is well established that the vertebrate photoreceptors are hyperpolarized when illuminated (Tomita, 1965; Kaneko & Hashimoto, 1967; Werblin & Dowling, 1969; Baylor & Fuortes, 1970). Since the polarity of the receptor potential is opposite to that of many other types of receptors, transmission of signals from the vertebrate photoreceptors to the second order neurones appears very peculiar. From an experiment of transretinal electrical stimulation, Trifonov (1968) suggested that a transmitter which depolarizes horizontal cells is continuously released in the dark and the release diminishes or stops when the receptors are hyperpolarized by illumination. This hypothesis has been supported by Toyoda, Nosaki & Tomita (1969) with the fact that the membrane conductance of *Necturus* horizontal cells was maintained at a high value in the dark and decreased by light. Experimental results by Dowling & Ripps (1973), Cervetto & Piccolino (1974) and Trifonov, Byzov & Chailahian (1974) are also in accord. In those experiments, horizontal cells are shown to be hyperpolarized in either low Ca or high Mg medium in which transmitter release is supposed to stop.

The aim of the present experiments is to further examine the mechanism of synaptic transmission from photoreceptors to horizontal cells. By blocking or facilitating the transmitter release, changes in the membrane potential of horizontal cells were recorded. Furthermore, by analysing the ionic dependence of horizontal cell membrane potential, ionic mechanisms of horizontal cell responses were also studied.

METHODS

Preparations. Carp, *Cyprinus carpio*, of about 500 g body wt., were light-adapted in an aerated, illuminated tank. The eyes were excised, cut at the equator, and the cornea and lens removed. After the vitreous humour was drained off with a hypodermic needle connected to a suction pump, the retina was isolated, receptor side up, on a piece of dry filter paper. The isolated retina was then transferred into a Teflon perfusion chamber 13 mm wide, 3 mm deep and with a capacity of 1·0 ml. (Fig. 1). The preparation was fixed on the bottom of the chamber by putting a pair of platinum claws on the edges of the filter paper.

Solutions. The retina was continuously perfused with 1·6 ml./min oxygenated Ringer solution. Rough estimation based on an exponential concentration change showed that with this speed of perfusion, 63 % of fluid was replaced in 50 sec. The normal Ringer solution contained (in mM): NaCl, 110; KCl, 2·5; $CaCl_2$ 2·2; glucose, 10; HEPES (N-2 hydroxyethylpiperazine N′-2 ethanesulfonic acid), 5·0 and pH was adjusted to 7·7 with NaOH. In another kind of Ringer HEPES was replaced by equimolar Tris buffer (tris hydroxymethyl aminomethane) adjusted to pH 7·7 with HCl. Both buffers worked equally satisfactorily and no difference was seen in the retinal responses. Ca-free solution contained (in mM): NaCl, 90; KCl, 2·5; $MgCl_2$, 20; glucose 10, and HEPES, 5: no Ca-chelating agent was added. In Na-free solution,

all Na was replaced with equimolar choline and Tris buffer was used. Chloride-free solution was made by replacing Cl with sulphate, and HEPES buffer was used. All solutions were made with glass-distilled water.

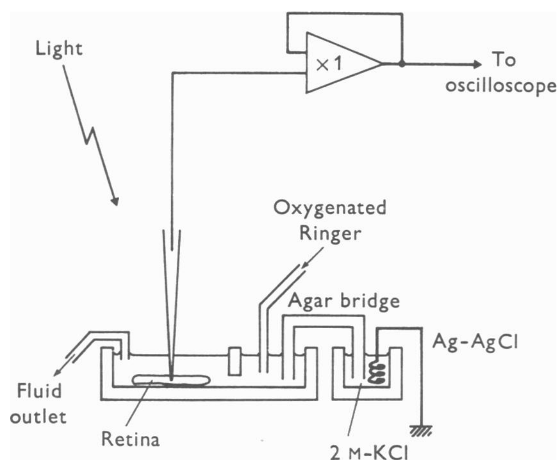

Fig. 1. Schematic diagram showing the set-up of experiments.

Recording electrodes. Receptors and horizontal cells were penetrated with glass micro-electrodes filled with 3 M-KCl and having a resistance of about 60–100 MΩ in the Ringer bath. The reference electrode was an Ag-AgCl, immersed in 2 M-KCl chamber which was connected to the main recording chamber with an agar bridge. The micro-electrode was connected to a high input impedance pre-amplifier and the electrical signals were observed on a cathode-ray oscilloscope, stored on FM magnetic tapes and simultaneously recorded on a chart recorder.

Photostimulator. Retinas were diffusely illuminated by a two-channel photostimulator which was described elsewhere (Tomita, Kaneko, Murakami & Pautler, 1967). Usually monochromatic lights of 520 and 620 nm with an intensity of 3×10^{12} quanta/mm^2 sec were given alternately. From the response polarity and amplitudes to these two monochromatic lights, one can identify the response types of horizontal cells. In the present experiments, those horizontal cells showing L-type responses (hyperpolarization to all wave-lengths) were selected.

Cell identification. Recorded cells were identified by the response type, based on the criteria previously established by correlating to the cell morphology (Kaneko, 1970); namely horizontal cells were characterized by graded, sustained responses with a large and uniform receptive field (S-potential). In experiments in which responses to light flashes were abolished, the cells were identified from the recording depth in the retina. This criterion was also useful and fairly reliable, since some samples were morphologically identified as horizontal cells by dye injection with Procion Yellow MX4R (Stretton & Kravitz, 1968).

All experiments were done at a room temperature of about 20° C and under a constant dim illumination of about 10 cd/m^2 to maintain the retina in the photopic state. This is the condition under which the carp rods are entirely suppressed (Kaneko & Yamada, 1972). Therefore, all horizontal cells studied in the present experiments were those which received inputs only from cones.

RESULTS

Responses from horizontal cells in the normal retina

In normal Ringer horizontal cells of a fresh retina showed membrane potentials of between −20 and −40 mV in reference to the bath (initial part of Fig. 3). In good preparations, the L-type horizontal cells had almost the same dark potential level. In response to light illumination they showed graded, sustained hyperpolarization. During the first 1 hr perfusion with normal Ringer, the membrane potential and the response amplitude remained almost unchanged. Most of the recordings were made

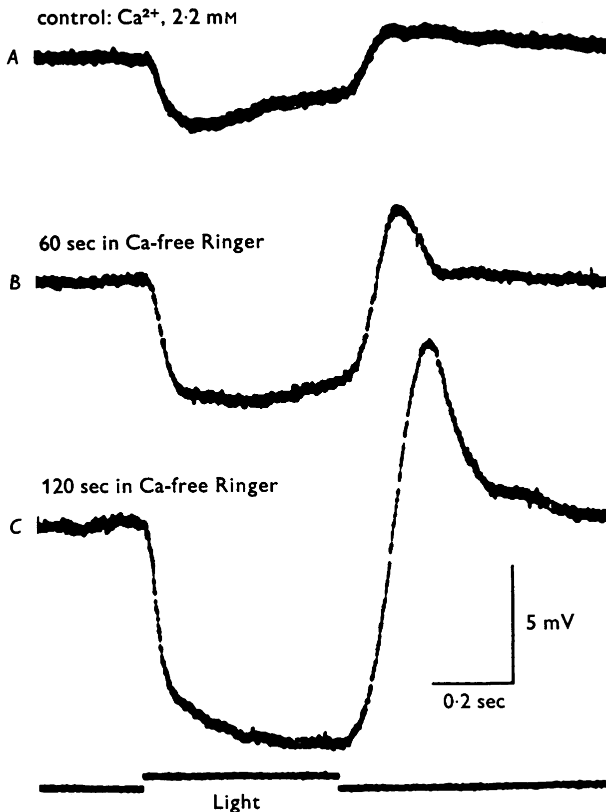

Fig. 2. Intracellular recording from a red-sensitive cone. Diffuse flashes of 620 nm, having 3×10^{12} photon.mm^{-2}.sec^{-1} were given repeatedly every 2 sec. (The same light intensity was used throughout the experiments.) *A*, in normal Ringer containing 2·2 mM-Ca and no Mg. *B*, 1 min after the perfusate was changed to a Ca-free test solution containing 20 mM-Mg. *C*, 2 min in Ca-free solution.

during this period. After about 2 hr, the membrane potential in the dark increased (hyperpolarized) and the response amplitude became smaller.

Electron microscopic observation of the retina perfused for 2 hr with the normal Ringer revealed no sign of deterioration. The lamellar structure of the rod and cone outer segments were well preserved and the photoreceptor terminal contained as many synaptic vesicles as the control preparation.

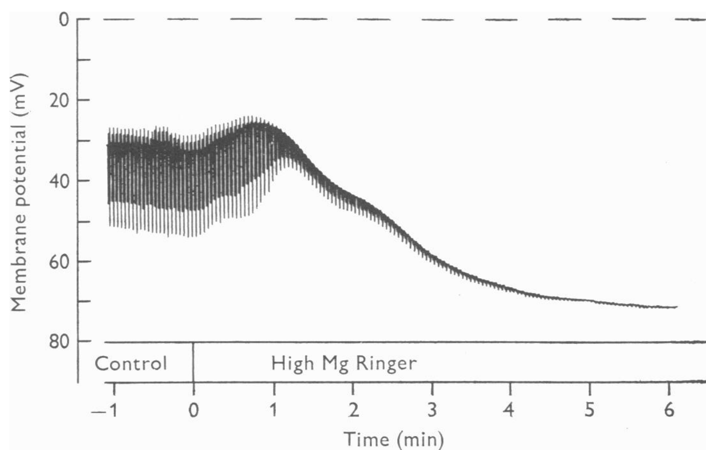

Fig. 3. Intracellular recording from a horizontal cell showing an L-type S-potential. Since the time scale is compressed, light responses are seen as vertical lines. Diffuse 620 and 520 nm flashes of equal intensity were given alternately. Red light gave larger responses. Zero on the time scale indicates the instance at which normal Ringer was switched to a Ca-free test solution containing 20 mM-Mg.

Effects of low Ca and high Mg on receptors and horizontal cells

Fig. 2 shows responses recorded from a red-sensitive cone in normal Ringer (*A*), 1 min (*B*) and 2 min (*C*) after changing the solution to a Ca-free medium. Contrary to horizontal cells, the receptor cell was depolarized in this test solution and its responses were strongly enhanced. A prominent 'off' rebound was seen at the cessation of illumination. The present results are in accord with the previous observation that low Ca enhanced Na permeability of receptor outer segments (Yoshikami & Hagins, 1973) and also with Brown & Pinto's experiment (1974) on single rods of the toad.

Calcium plays an important role also for the transmitter release from the presynaptic terminals. Here, Ca-free solution was used to block the chemical transmission from photoreceptors to horizontal cells. Fig. 3 shows a continuous record from an L-type horizontal cell. After switching the bathing medium from normal Ringer to Ca-free test solution, the

horizontal cell was slightly depolarized at first, but soon gradually hyperpolarized until the membrane potential reached −70 mV level in about 5 min. With hyperpolarization, the light responses completely disappeared. In Ca-free medium all horizontal cells showed a large membrane potential of around −80 mV and no light responses.

Most of these cells were identified from the recording depth in the retina. To confirm this identification, ten such units were injected with Procion Yellow MX4R from the recording electrodes: four of them were morphologically determined as external horizontal cells and six were internal horizontal cells.

Comparing the behaviour of cones and horizontal cells, it seems likely that the hyperpolarization of horizontal cells is a result of interruption of the synapse between the receptor and horizontal cells, since the membrane potential of horizontal cells did not follow the changes of the receptors.

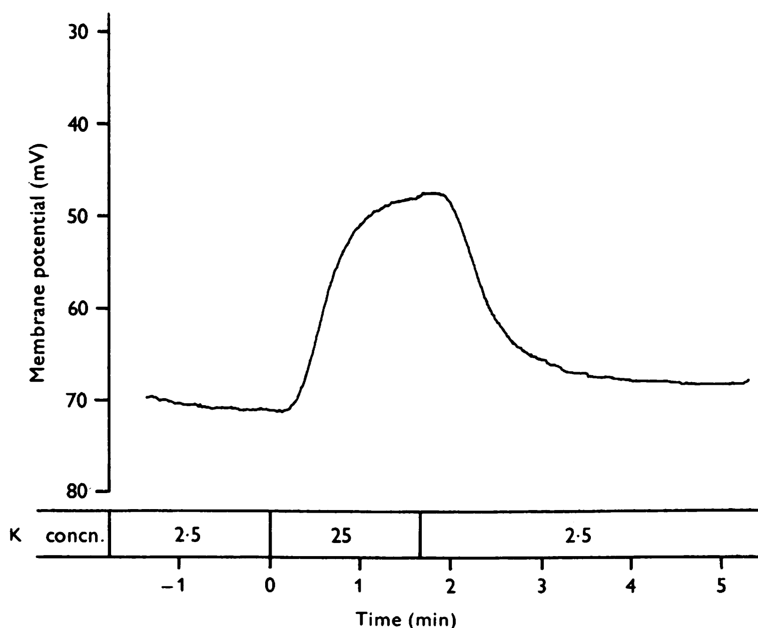

Fig. 4. Membrane potential of a horizontal cell after its synaptic input was cut off by perfusing the retina with a Ca-free test solution containing 20 mM-Mg. A test solution containing 25 mM-K (Na was replaced by K; other composition remained the same) was applied from 0 min to 1⅔ min.

Effects of K on the horizontal cell membrane potential

In the preceding experiment it was shown that horizontal cells show a high membrane potential after the synaptic input was removed. It is widely found in various neurones that K ions are responsible for the resting

membrane potential. In this experiment, horizontal cells, already deprived of synaptic inputs, were perfused with test solutions containing various concentrations of K (NaCl was replaced by KCl). Fig. 4 shows an example of such records. Since the synapse has already been blocked by Ca-free Ringer, the horizontal cell membrane potential was about -70 mV and no light responses were seen. Tenfold increase of K in the perfusate produced a prompt depolarization of about 25 mV. By returning the K concentration to the control value, the membrane potential returned almost to the previous level with a similar time course as that of depolarization.

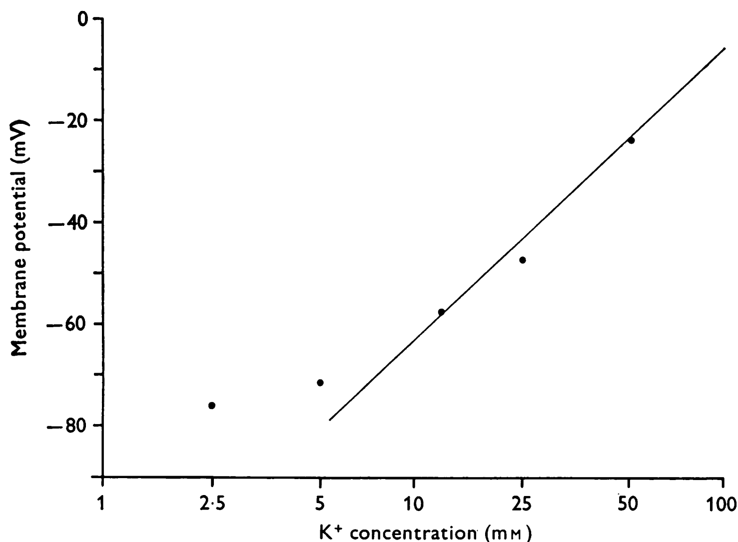

Fig. 5. Relationship between the membrane potential of a horizontal cell and the external K concentration, after the synapses had been blocked by a Ca-free high Mg solution. The continuous line indicates the theoretical relationship calculated by the Nernst equation. Points represent membrane potentials obtained from one and the same horizontal cell.

These results indicate that the horizontal cells are sensitive to the external K concentration, and that small ions like K have relatively free movement in the retina, making an equilibrium within a few minutes.

The relationship between the membrane potential and the K concentration was determined and shown graphically in Fig. 5. The continuous line indicates the calculated relationship based on the Nernst equation with a slope of 58 mV per decade, and on an assumption that the intracellular K concentration was 125 mM. Points indicate membrane potentials measured in one and the same horizontal cell. At higher concentrations (12·5 mM and more), the membrane potentials were in good agreement with the

calculated value. At lower concentrations (5 mM and less), the membrane potential was lower than the hypothetical value. This is presumably because of a leakage of the cell membrane produced by a damage from electrode penetration or by permeability to other kinds of ions, such as Na, which has been observed in other nerve cells. In the normal Ringer, tenfold increase of external K produced only slight depolarization, contrary to the similar experiment in Ca-free Ringer. It therefore seems probable that in normal conditions other kinds of ions than K are making more contribution to determining the dark membrane potential of horizontal cells. In Ca-free Ringer, changes in either Na or Cl concentrations produced little change in the horizontal cell membrane potentials.

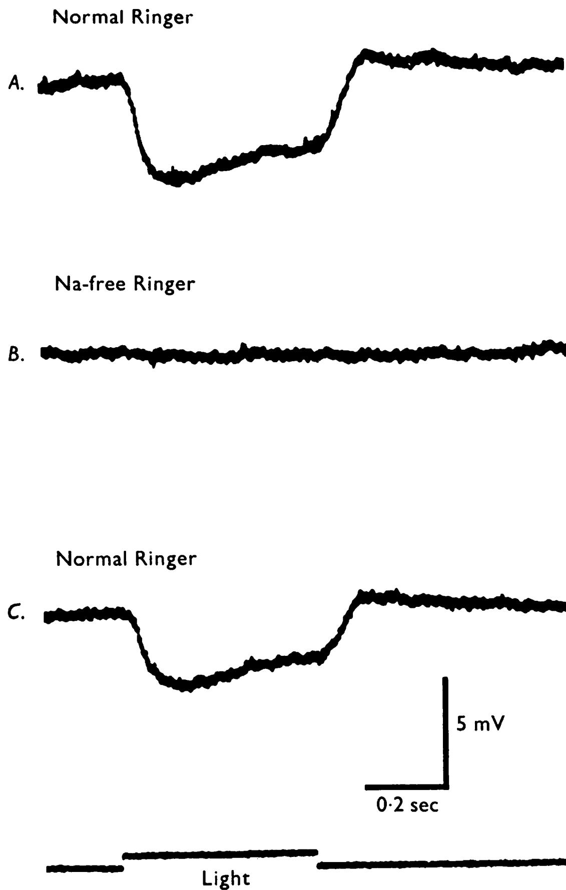

Fig. 6. Responses of a red-sensitive cone to diffuse 620 nm flashes (the same cell as in Fig. 2). *A*, in normal Ringer. *B*, 1 min after switching the perfusate to Na-free choline Ringer. *C*, 1 min after reapplying normal Ringer.

Effects of Na on the membrane potential of the receptors and horizontal cells

The membrane potential and the response of cones were highly dependent on the external Na concentration. Fig. 6 shows responses of a red-sensitive cone in normal Ringer (*A*) and in Na-free test solution (*B*). The dark membrane potential of this cone promptly increased (hyperpolarized) and responses were lost in the Na-free medium. On reapplication of normal Ringer, the membrane potential and responses to light flashes quickly recovered.

The results of Fig. 6 are in accord with the similar observation on the toad rods (Brown & Pinto, 1974) and on the turtle cones (Cervetto, 1973), indicating that the photoreceptors maintain a high Na permeability in the dark and the illumination reduced Na permeability.

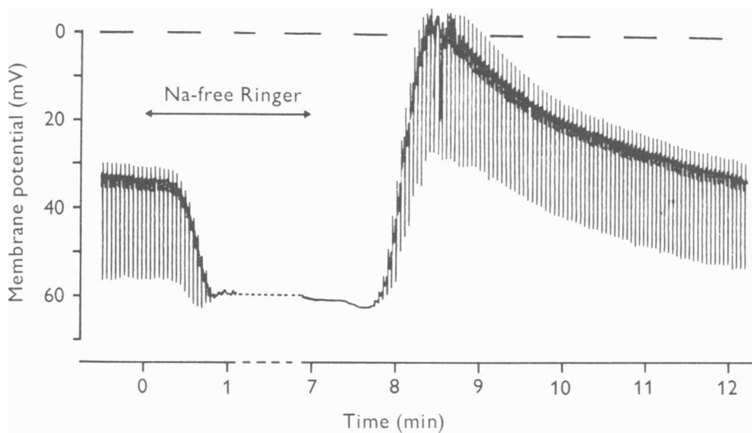

Fig. 7. Intracellular recording from an L-type horizontal cell. In this experiment, only 620 nm flashes were used. At 0 min normal Ringer was switched to Na-free Ringer. At 7 min normal Ringer was reapplied to the retina. Since no membrane potential changes were seen between 1–7 min of perfusion with Na-free medium, this part of the record was cut out and shown with a dashed line.

Removal of Na from the perfusate also hyperpolarized horizontal cells. Fig. 7 shows one of such experiments. By switching the perfusate from normal Ringer to the Na-free test solution, the dark membrane potential became about -60 mV and light responses disappeared. After about 7 min in Na-free solution, the normal Ringer was reapplied, and then the horizontal cell was again depolarized and the light responses reappeared. The depolarization reached its maximum in about $1\frac{1}{2}$ min and then gradually returned to the initial level. At the maximum depolarization the membrane potential transiently crossed zero level (potential reversal),

making the inside of the cell positive in reference to the extracellular space. Although, unfortunately, Fig. 6 does not show a continuous change, the time course of the membrane potential changes in the horizontal cell roughly corresponded to those in the receptor. Although the hyperpolarization of horizontal cells in Na-free medium may be the secondary effect via cones, it seems likely that the transmitter increased Na permeability of horizontal cell membrane. This assumption is supported by the transient membrane potential reversal which was produced probably by a larger concentration gradient of Na across the horizontal cell membrane, after a loss of intracellular Na during perfusing with Na-free medium.

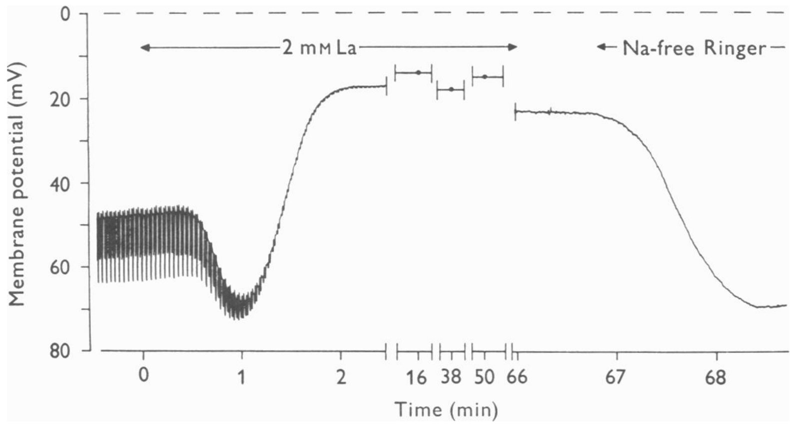

Fig. 8. Intracellular recordings obtained from five horizontal cells of the same preparation, and combined to make a continuous record. Each broken part represents a record from single cells. Diffuse light flashes of 520 and 620 nm were given alternately. Short vertical lines are responses to green flashes and long ones to red. Test solution containing 2 mM-La was applied from 0 to 66 min. At 66 min normal Ringer was reapplied. At 67 min it was switched to Na-free Ringer.

Effects of La on receptor and horizontal cells

In the neuromuscular junction La ions are shown to accelerate the transmitter release from the presynaptic terminal (Heuser & Miledi, 1971), and here La was applied to the retinal photoreceptors. Fig. 8 shows a continuous record from horizontal cells. On applying Ringer containing 2 mM-La, the horizontal cell was transiently hyperpolarized initially, but soon it was strongly depolarized to a potential level between −15 and −20 mV. Responses to light flashes almost disappeared with membrane depolarization. This depolarization was maintained for many hours. Unfortunately since this cell was lost 3 min after starting La perfusion, several other horizontal cells were penetrated in the same preparation. All of these

cells showed similar membrane potential during La perfusion. At 66 min of La perfusion, normal Ringer was reapplied to the retina, but horizontal cells remained still depolarized. It is unlikely, however, that this prolonged depolarization is a sign of cell deterioration, since application of Na-free Ringer quickly hyperpolarized the horizontal cell to -70 mV. Similar effects were seen with 0·1 mM-La, but with a slower time course.

Contrary to horizontal cells, responses from cones remained almost unchanged during La perfusion. Cone responses were often recorded from the retina perfused with La even after 1–2 hr perfusion.

From these results, it is tempting to conclude that La facilitated the transmitter release also from photoreceptor terminals, causing depolarization of horizontal cells. Probably the effects persisted even after La was washed away. Hyperpolarization in Na-free Ringer indicates again that the transmitter increased Na permeability of horizontal cell membrane.

DISCUSSION

Synaptic inputs to horizontal cells

It is well established that Ca ions are playing an important role in the release of neural transmitters (cf. Katz, 1969). Furthermore in the vertebrate photoreceptors, Ca has been suggested as a mediator between the visual excitation at photopigment molecules and the conductance change occurring at the cell membrane (Yoshikami & Hagins, 1973; Brown & Pinto, 1974). The dual action of Ca makes the present results slightly complicated.

In the carp cones low Ca depolarized the membrane and enhanced light responses, as in the turtle cones and toad rods (Cervetto & Piccolino, 1974; Brown & Pinto, 1974). This may be the result of Ca deprivation from the outer segment of cones. Horizontal cells showed entirely opposite behaviour; hyperpolarization of the membrane potential and suppression of responses. It seems likely, therefore, that changes in horizontal cells is the result of reduction of transmitter release from the photoreceptor cells. Certainly, while the synaptic transmission was still working, parallel changes were observed between cones and horizontal cells (Fig. 3). Horizontal cells were slightly depolarized and responses enhanced soon after the application of Ca-free solution.

Cones showed a prominent 'off' rebound in Ca-free medium. The nature of this response was not studied systematically, but it can be pointed out that this enhancement of 'off' response is not the result of interaction with other cells, since the 'off' response persisted after the chemical transmission had been blocked (cf. Fig. 3). It rather appeared to be produced by a change in the cone membrane properties by the removal of Ca.

Dependence on K of membrane potential of horizontal cells after synaptic block

When the synaptic transmission was blocked, the membrane potential of horizontal cells was highly dependent on external K. The present experiments with perfusate containing various concentrations of K showed that the membrane potential agreed with the K equilibrium potential (E_K). Furthermore, changes in Na and Cl concentrations showed little effect. It seems safe to conclude that free from the influence of transmitter substance, the membrane potential of horizontal cells is determined by E_K and that the hyperpolarizing responses of horizontal cells in the normal Ringer is produced by the membrane potential approaching E_K.

Ionic mechanism of horizontal cell depolarization in the dark

It has been suggested by several authors that the endogenous transmitter from photoreceptors depolarizes horizontal cells (Trifonov, 1968; Toyoda *et al.* 1969; Dowling & Ripps, 1973; Cervetto & Piccolino, 1974). Naturally a question arises concerning the mechanisms of horizontal cell depolarization in the dark. Although Na is the most likely candidate, simple change in external Na will not give us a satisfactory answer. Since the membrane potential of cones is highly dependent on the external Na, a change in Na concentration always affected cones first, and then secondarily horizontal cells. Fortunately, a few hints were found in the present experiments suggesting Na as the major ion contributing to horizontal cell depolarization. The first was the transient membrane potential reversal of horizontal cells when the normal Ringer was reapplied to the retina after it had been perfused with Na-free medium (Fig. 7). This reversal cannot occur unless the membrane is permeable to Na. Probably by the perfusion with Na-free medium, the intracellular Na concentration was reduced. On return to the normal Ringer, E_{Na} was more positive than in the control state. These results indicate that the horizontal cell membrane maintains a high Na permeability in the dark under the influence of the endogenous transmitter.

Another hint was found in the experiment of Fig. 8. Here, removal of external Na quickly hyperpolarized horizontal cells which remained depolarized under the residual influence of La, indicating again a high permeability produced by the endogenous transmitter.

Mechanisms of S-potential generation

The present results are in accord with a hypothesis, originally presented by Trifonov (1968) and supported by Toyoda *et al.* (1969), that in the dark

a transmitter is continuously liberated from the receptor terminals, and the transmitter depolarizes horizontal cell membrane.

As summarized in the schema of Fig. 9, horizontal cell is maintained in depolarization in the dark by a high conductance (expressed by the closed switch) in series with E_D. E_D indicates equilibrium potential of the dark membrane potential to which Na is contributing significantly. On illumination, R_D increases (expressed by the open switch) and then the membrane potential is determined by E_K. This schema gives a satisfactory interpretation of the present experiments.

More direct analysis of the ionic mechanisms of horizontal cell responses will be made by applying an identified transmitter substance of the photoreceptor. Unfortunately one has to wait for the reliable transmitter identification in the future.

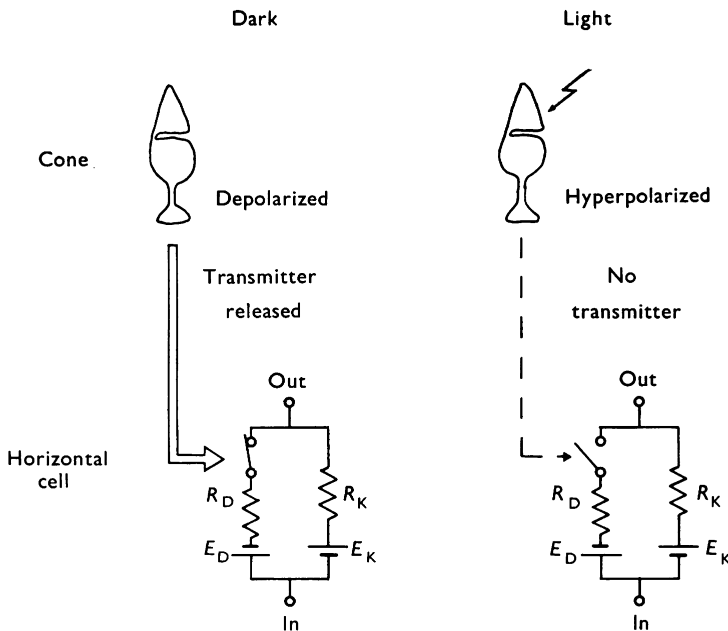

Fig. 9. Schematic diagram showing a model proposed to interpret the relationship between the light responses of cones and the transmitter release and the effects of transmitter substance on the horizontal cell membrane. The lower circuit diagram represents equivalent circuit of horizontal cell membrane. Changes in R_D should be gradual, but for simplicity, it is shown by opening (R_D increase) and closing (R_D decrease) the switch. E_D, equilibrium potential of the dark membrane potential; E_K, K equilibrium potential.

We are indebted to Dr M. Murakami for steady encouragement and discussions. This work was supported in part by a grant from the Japanese Education Ministry (No. 911114), by Bionics Research Grant from the Japanese Ministry of International Trade and Industry and by U.S. PHS grant No. EY00017 (T. Tomita, Principal Investigator).

REFERENCES

BAYLOR, D. A. & FUORTES, M. G. F. (1970). Electrical responses of single cones in the retina of the turtle. *J. Physiol.* **207**, 77–92.

BROWN, J. E. & PINTO, L. H. (1974). Ionic mechanisms for the photoreceptor potential of the retina of *Bufo marinus*. *J. Physiol.* **236**, 575–591.

CERVETTO, L. (1973). Influence of sodium, potassium and chloride ions on the intracellular responses of turtle photoreceptors. *Nature, Lond.* **241**, 401–403.

CERVETTO, L. & PICCOLINO, M. (1974). Synaptic transmission between photoreceptors and horizontal cells in the turtle retina. *Science, N.Y.* **183**, 417–419.

DOWLING, J. E. & RIPPS, H. (1973). Effects of magnesium on horizontal cell activity in the skate retina. *Nature, Lond.* **242**, 101–103.

HEUSER, J. & MILEDI, R. (1971). Effect of lanthanum ions on function and structure of frog neuromuscular junctions. *Proc. R. Soc.* B **179**, 247–260.

KANEKO, A. (1970). Physiological and morphological identification of horizontal, bipolar and amacrine cells in goldfish retina. *J. Physiol.* **207**, 623–633.

KANEKO, A. & HASHIMOTO, H. (1967). Recording site of the single cone response determined by an electrode marking technique. *Vision Res.* **7**, 847–851.

KANEKO, A. & YAMADA, M. (1972). S-potentials from the dark-adapted retina of the carp. *J. Physiol.* **227**, 261–273.

KATZ, B. (1969). *The Release of Neural Transmitter Substances*. Liverpool: Liverpool University Press.

STRETTON, A. O. W. & KRAVITZ, E. A. (1968). Neuronal geometry: determination with a technique of intracellular dye injection. *Science, N.Y.* **162**, 132–134.

TOMITA, T. (1965). Electrophysiological study of the mechanisms subserving color coding in the fish retina. *Cold Spring Harb. Symp. quant. Biol.* **30**, 559–566.

TOMITA, T., KANEKO, A., MURAKAMI, M. & PAUTLER, E. L. (1967). Spectral response curves of single cones in the carp. *Vision Res.* **7**, 519–531.

TOYODA, J.-I., NOSAKI, H. & TOMITA, T. (1969). Light-induced resistance changes in single photoreceptors of *Necturus* and *Gekko*. *Vision Res.* **9**, 453–463.

TRIFONOV, YU. A. (1968). Study of synaptic transmission between photoreceptors and horizontal cells by means of electric stimulation of the retina (in Russian). *Biofizika* **13**, 809–817.

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

WERBLIN, F. S. & DOWLING, J. E. (1969). Organization of the retina of the mudpuppy, *Necturus maculosus*. II. Intracellular recording. *J. Neurophysiol.* **32**, 339–355.

YOSHIKAMI, S. & HAGINS, W. A. (1973). Control of the dark current in vertebrate rods and cones. In *Biochemistry and Physiology of Visual Pigments*, ed. LANGER, H., pp. 245–255. Berlin: Springer-Verlag.