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EFFECTS OF CHEMICALS ON RECEPTORS AND HORIZONTAL CELLS IN THE RETINA

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SUMMARY

1. By applying atomized chemical solutions on to gecko and carp retinae, neuropharmacological reactions of the photoreceptors and horizontal cells were observed.

2. Sodium L-glutamate and L-aspartate, glycine, ACh and GABA, had no appreciable effect on the photoreceptor activity.

3. Carp horizontal cells were depolarized by both L-glutamate and L-aspartate. When maximally depolarized, the action of the endogenous transmitter from the receptor terminals was completely masked, resulting in abolition of the S-potentials.

4. Responses to red light in both L- and C-type horizontal cells were more strongly affected by aspartate than responses to blue light.

5. Glycine and GABA hyperpolarized the horizontal cells, and the S-potentials were diminished.

6. ACh had no effect on the activity of the horizontal cells.

INTRODUCTION

Electron microscopic studies have shown that photoreceptors make synaptic contacts with bipolar and horizontal cells (Stell, 1967; Dowling & Werblin, 1969; Witkovsky & Dowling, 1969). The structure of the synapse is provided with synaptic vesicles, synaptic cleft, etc., which suggest that transmission is chemically mediated. In the fish retina, the correlations between the three types of cells involved and the characteristics of their electrical responses are well established (Kaneko, 1971). The photoreceptors respond to light with hyperpolarization, as a consequence of being partially depolarized in the dark and polarized in the light (Toyoda,

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Nosaki & Tomita, 1969; Tomita, 1970). The horizontal cells give rise to the S-potentials which fall into two classes: the luminosity type (L-type) and the chromaticity type (C-type). In the L-type, the response is a hyperpolarization to any wave-length of light, while in the C-type the sign of the response is wave-length-dependent (MacNichol & Svaetichin, 1958; Tomita, 1965). The bipolar cells have centre-surround receptive field organization (Kaneko, 1970). These observations suggest that the mechanisms of the synapse are highly elaborate. A hypothesis has been proposed by Trifonov (1968) and by Toyoda *et al.* (1969): an excitatory transmitter in the receptor terminals is continuously liberated in the dark and ceases to be released in the light, causing the horizontal cell to hyperpolarize. This explanation applies only to the L-type S-potential. The mechanisms for synaptic transmission are not completely resolved and no chemical transmitter has been identified (cf. Lolley, 1969; Witkovsky, 1971).

One method to study synaptic transmission is to observe alterations in behaviour of post-synaptic cells caused by externally applied neuropharmacological agents. Horizontal cells, on which our observations were made in these experiments, respond to light only with graded slow potentials and consequently intracellular recording is essential for the detection of their electrical changes. The usual techniques of chemical application such as perfusion or microiontophoresis are difficult to use because of the dislodgement of recording electrodes or because of the small size of the retinal cells. Accordingly a method for application of atomized chemical solution on to the retina was developed.

This paper will describe our observations on the effects of several chemicals on horizontal cell activity and the electroretinogram (e.r.g.).

METHODS

Materials and potential recording techniques

Isolation of the mass response of the receptor potential from the other e.r.g. components was carried out on the isolated inverted retina of the carp, *Cyprinus carpio*, using techniques described elsewhere (Murakami & Kaneko, 1966; Murakami & Sasaki, 1968). In short, the outer pipette of a coaxial micro-electrode (Tomita, 1962) was located at the distal margin of the isolated retina, and the inner pipette was protruded from the tip of the outer pipette into the retina to a depth of about 120 μm (Fig. 1). Differential recording between the outer and inner pipettes (Channel I in Fig. 1) gave responses arising in the receptor cell layer (receptor potential) and potentials recorded between the inner pipette and the indifferent electrode (Channel II) were compound responses of PII and proximal PIII (Murakami & Sasaki, 1968). Both kinds of responses were led to the oscilloscope through CR-coupled preamplifiers with a time constant of 1·0 sec.

For intracellular recording from receptor cells, isolated retinae of the Tokay gecko, *Gekko gekko*, were used because of its large photoreceptor cells. The dark-adapted eye was enucleated under dim red light, and the isolated retina was mounted

receptor-side up on a jolting device (Tomita, Kaneko, Murakami & Pautler, 1967). Penetration of receptor cells with a micro-electrode was accomplished by a technique described by Tomita (1965) and by Toyoda *et al.* (1969). The intracellular micro-electrodes were glass pipettes filled with 3 M-KCl solution.

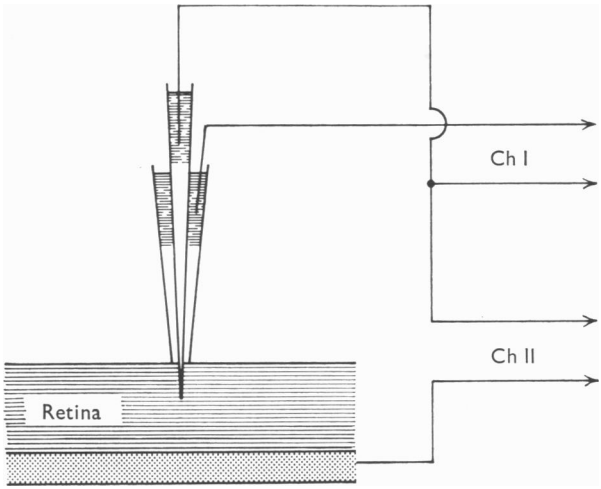

Fig. 1. Arrangement for isolation of the receptor potential from the other e.r.g. components with a coaxial micro-electrode in the isolated inverted carp retina. Channel (Ch) I records the potential difference between the inner and outer pipettes, and Channel (Ch) II the potential between the inner pipette and the indifferent electrode (Murakami & Kaneko, 1966).

For intracellular recording from horizontal cells, isolated retinae of the carp were used. The retina was mounted receptor-side up in a small dish made of Teflon ring on a glass plate. Then, the retina on the dish was placed in a water jacket chamber attached to the jolting device.

The two-channel photostimulator used has been described elsewhere (Tomita *et al.* 1967).

The temperature of the retina was maintained near 15° C by means of a water jacket in which cooled water was circulated.

Application of chemicals

Atomized chemical solutions were sprayed on the receptor surface of the retina. In the isolated retina mounted receptor-side up, diffusion is rapid and the neurones were readily accessible to chemicals (Murakami & Sasaki, 1968). Fig. 2 is a sketch of the experimental arrangement which consists of two nebulizing systems enabling successive applications of two different chemicals. Application of chemicals was controlled by a cock (C in Fig. 2) operated manually. With the cock in the neutral position, humidified air was blown directly through the cock to the water jacket chamber. To apply a chemical, the cock was turned so that the air passed first through either of the ultrasonic nebulizers, A and B, where it picked up an atomized chemical solution, and then carried it into the water jacket chamber. Any spray of chemicals overflowing the chamber during application was removed through a filter of activated carbon. A fine bifurcate pipe-line (BP) bypassing the cock and nebulizers was used to push back contaminating chemical solutions coming from the working nebulizer to the one at rest.

Operation of the cock for chemical application was monitored on the oscilloscope. Actual arrival and cut-off times of chemicals at the water jacket chamber were delayed about 2 sec after the monitoring signs.

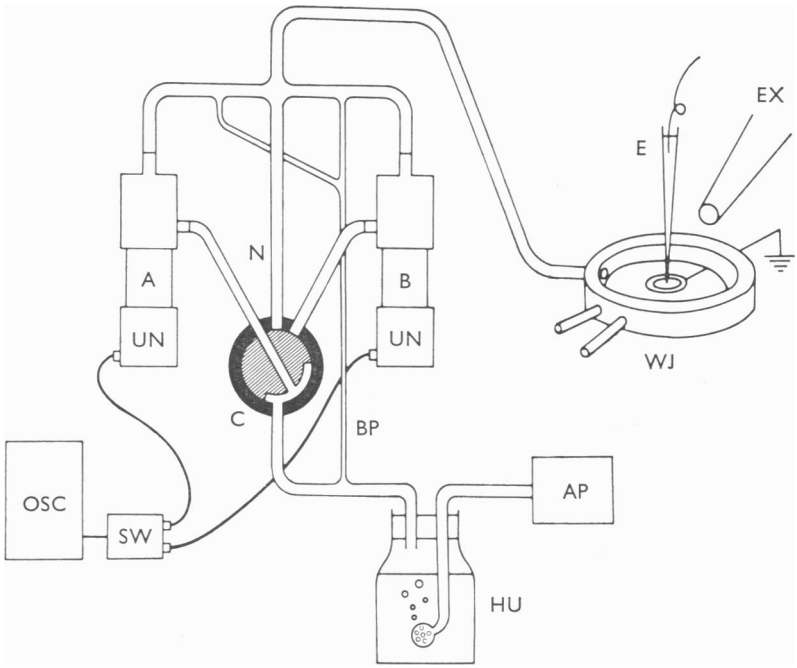

Fig. 2. The assembly for application of atomized chemical solutions on to the retina. AP: air pump; HU: humidifier; C: cock; UN A and B: ultrasonic nebulizers; OSC: electronic oscillator; N: neutral position of the cock; E: glass micropipette electrode; WJ: water jacket; EX: exhaust. Further explanation in text.

Chemicals

The following chemicals were used in these experiments: sodium L-glutamate, sodium L-aspartate, glycine, γ-aminobutyric acid (GABA), and acetylcholine chloride (ACh): all commercially available, reagent grade. In these experiments, only the L-isomers of glutamate and aspartate were used, so that they will be referred to simply as glutamate and aspartate hereafter.

Measurement of the amount of amino acid applied on the retina

The amount of amino acid administered on the retina was measured by the ninhydrin method. A piece of filter paper, moistened with distilled water, was placed in the water jacket chamber in Fig. 1, and a 1 M solution of amino acid, sodium glutamate or aspartate, was sprayed for 10 sec. Pieces of paper were collected into distilled water and the amount of substance was measured. The average of three trials for sodium glutamate was calculated as a rate of 0·18 μmole falling on to an area of 1 cm^2 per 10 sec, and 0·11 μmole/cm^2 per 10 sec for sodium aspartate.

The concentration of each chemical in the retinal tissue itself, however, was unknown. Very roughly, it could be estimated on the assumption that these chemicals diffused uniformly into the retinal tissue, but were prevented from further diffusion

into the vitreous humour by the inner limiting membrane, which is known to have a low permeability (Murkami & Sasaki, 1968). Concentration of these amino acids in the retina were approximately 7·3 mM for glutamate and 4·5 mM for aspartate. These values corresponded to those sufficient to suppress the b-wave of the frog e.r.g. (Hanawa & Tateishi, 1970; also see Discussion).

The concentrations of chemicals other than glutamate and aspartate were estimated to be several mM in the retina on the assumption that the amount of chemical falling on the retina per unit time was proportional to the concentration of the chemical solution in the nebulizer.

RESULTS

Effects of chemicals on e.r.g. components

Effects of glutamate and aspartate

Isolation of the receptor potential from the other e.r.g. components was achieved with the experimental arrangement shown in Fig. 1, and the records are shown in Fig. 3. First, a small retinal area (2 mm diam.) around the recording electrode was illuminated with white light (focal illumination) through one channel of the photostimulator. Following a short interval after the cessation of focal illumination, the surrounding area of the retina was stimulated by white light (non-focal illumination) through the other channel. Fig. 3*a*–1 shows the isolated mass response of the receptor (distal PIII) recorded through Channel I in Fig. 1. This was positive with focal illumination, and also, because of light scattering, with non-focal illumination (Murakami & Sasaki, 1968). Fig. 3*a*–2 shows the other e.r.g. components, the compound response of PII and proximal PIII, recorded through Channel II in Fig. 1. The response to focal illumination showed positive polarity, which resulted from the predominance of proximal PIII, and to non-focal illumination with negative polarity because of PII (Murakami & Sasaki, 1968). The three e.r.g. components were thus isolated so that effects of chemicals could be observed separately on each.

After sodium glutamate was applied on the retina for about 10 sec, a remarkable diminution of PII and proximal PIII was observed (Fig. 3*b*–2), while the receptor potential still remained (Fig. 3*b*–1). Afterwards, the responses on record 3*b*–2 gradually recovered (3*c*–2), recovery being almost complete 80 sec after cessation of application (Fig. 3*d*–2). No appreciable difference was observed in the effect of the chemical between PII and proximal PIII.

Almost identical results were obtained with sodium aspartate.

Effects of glycine and GABA

The effect of glycine was observed using the same experimental arrangement as described above. After the control had been recorded (Fig. 4*a*),

glycine was applied for about 10 sec. No appreciable change in responses was observed (Fig. 4*b*). A further 10 sec application was then given and after 20 sec, Fig. 4*c* was recorded, where it is clear that PII was depressed more strongly than proximal PIII. The diminution of these responses

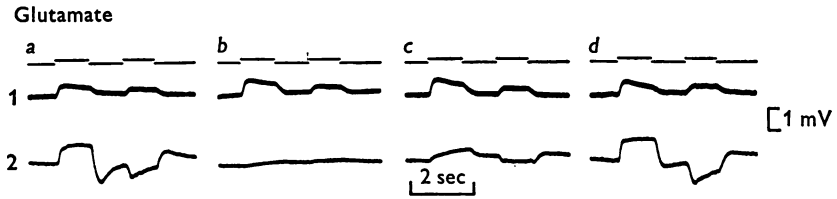

Fig. 3. Effect of glutamate on the e.r.g. components isolated by the arrangement in Fig. 1. Elevation of uppermost trace indicates light stimuli. Sequence of stimulation is described in text. Trace 1 of each record is the isolated receptor potential. Trace 2 is the compound response of PII and proximal PIII. Traces 1 and 2 are recorded with CR-coupled amplifiers with a time constant of 1·0 sec. *a*: control before application of glutamate, *b*: 20 sec after cessation of application, *c*: after 70 sec, *d*: after 80 sec.

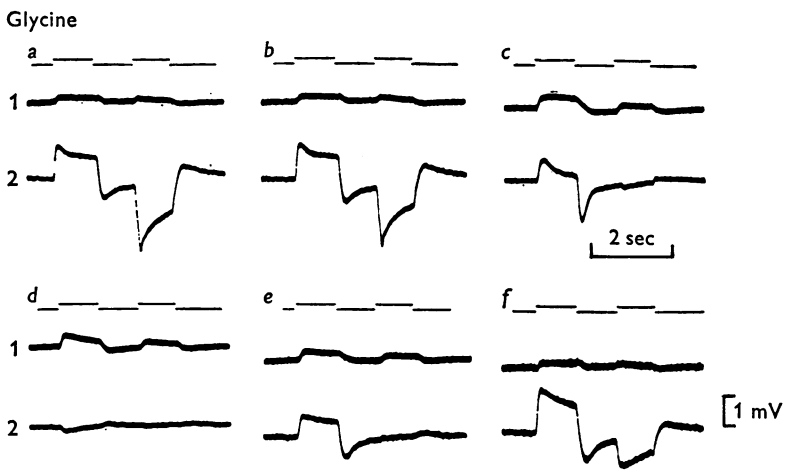

Fig. 4. Effect of glycine on the e.r.g. components. The traces correspond to those in Fig. 3. *a*: before application of glycine, *b*: after first application, *c*: after second application, *d*: 2 min, *e*: 5·7 min, *f*: 20 min after second application.

slowly progressed, leaving the receptor potential unchanged (*d*: 2 min after the second application). Thereafter, the responses began to recover (*e*: after 5·7 min, *f*: 20 min after the second application); however, complete recovery of PII was never observed. The time course of the effect of glycine is apparently slower than that of glutamate or aspartate.

GABA had a similar effect to that of glycine on the e.r.g. components.

Effect of ACh

ACh had no effect on any component of e.r.g., even at concentrations estimated to be as high as several mM.

Effects of chemicals on the receptor potential

To confirm that the chemicals have no appreciable effect on receptors, intracellular recording from gecko rods were made.

Effects of glutamate and aspartate

Isolated inverted retinae of the gecko were penetrated with glass micropipette electrodes. In a good preparation, the photoreceptor cells were readily penetrated at the moment the electrode touched the surface of the retina or when the retina was jolted. Hyperpolarizing responses were observed during illumination. Fig. 5*a* shows that repeated applications of sodium aspartate have no effect on the response although they cause a

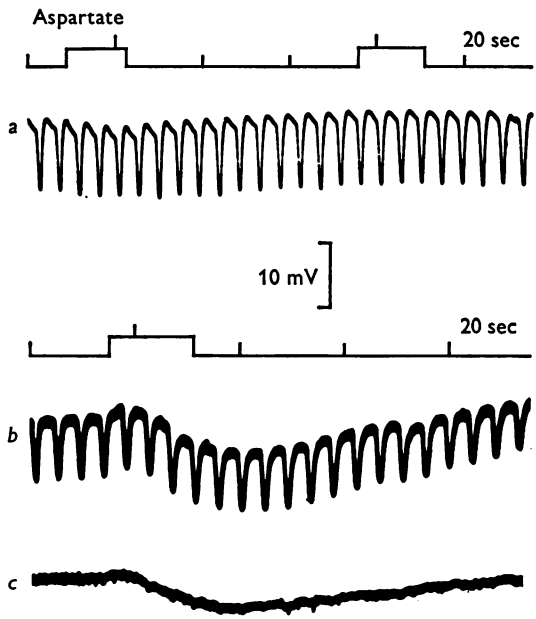

Fig. 5. Effect of aspartate on the photoreceptor activity of the gecko.
a: a negative-going d.c. shift of the base line superimposed on the intracellularly recorded receptor potential caused by aspartate.
b and *c*: the negative d.c. shift seen in record *a* is shown to arise extracellularly between the receptor cell and the indifferent electrode.
b: intracellular recording of the photoreceptor potential.
c: extracellular recording with the electrode withdrawn from the cell where record *b* was taken.

negative-going d.c. shift of the base line. Fig. 5*b* and 5*c* show evidence that this shift was not due to a change of the receptor potential itself, but rather to a potential change in structures other than the receptor cell. After the intracellular response was recorded (Fig. 5*b*), the electrode was withdrawn to the outside of the cell, where no response to light was detected. Here, a d.c. shift caused by the same amount of aspartate was recorded without light stimulation (Fig. 5*c*). The time courses of the base line shift in Figs. 5*b* and 5*c* are so similar that it appears that the d.c. shift superimposed on the intracellular recording is due to changes in the field potential originating in the neural element or elements intervening between the inner pipette and the indifferent electrode.

Effects of glycine, ACh and GABA

These chemicals also had no appreciable effect on the receptor potentials.

Effects of chemicals on S-potential

Effect of aspartate and glutamate

The isolated retina of the carp was diffusely illuminated alternately with blue (480 nm) and red (680 nm) lights. The wave-lengths were such that two types of S-potentials were readily distinguished: in the L-type, the response is a hyperpolarization to both wave-lengths, while in C-type the sign of the response is negative to blue and positive to red light (see Introduction). The S-potential shown in Fig. 6 was an L-type response. After a 6·5 sec application of sodium aspartate, the cell was rapidly depolarized to a level very close to, but never beyond, the extracellular level measured later by withdrawal of the electrode tip out of the cell. No response was seen as long as the cell was fully depolarized. During recovery, the responses to red light were temporarily smaller than those to blue light, suggesting that the mechanism generating the red response is more susceptible to aspartate than that governing the blue response.

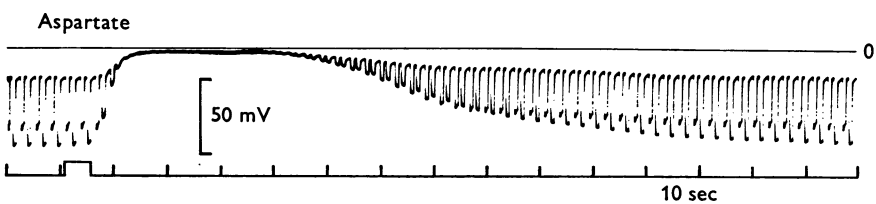

Fig. 6. Effect of aspartate on an L-type horizontal cell. A pair of monochromatic light stimuli (480 and 680 nm, adjusted to equal quantum flux) is given repetitively. Elevation of time scale indicates time of application of aspartate. The straight line indicated by 0 shows the extracellular potential level measured later by withdrawal of the electrode out of the cell.

In our experience, a cell with a dark level (potential level in the dark) ranging from -20 to -30 mV gives rise to a large S-potential, but a cell with unusually deep dark level does not. In the case shown in Fig. 7, a dark level of -58 mV was first recorded and the amplitudes of the S-potentials to blue and red lights were only a few mV. With application of sodium aspartate, the cell was rapidly depolarized, and at the peak, no response was observed. Around midway in recovery the S-potentials temporarily increased their amplitudes and a difference between the responses to blue and red lights was apparent. As a repolarization of the dark level progressed, thereafter, the amplitudes of the S-potentials became smaller and reached the control level.

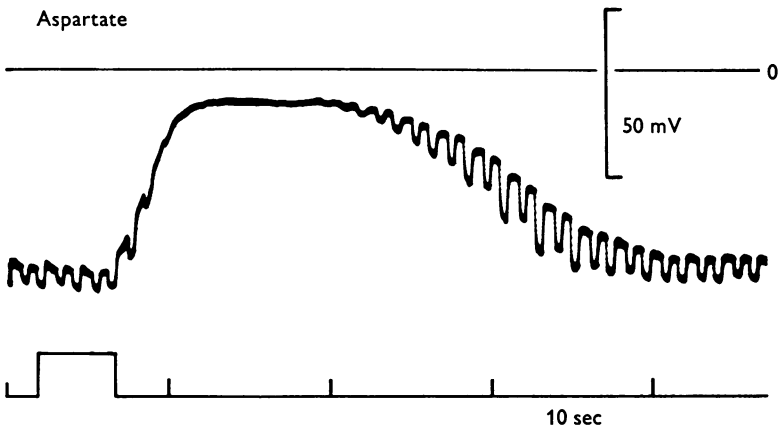

Fig. 7. Effect of aspartate on an L-type horizontal cell whose dark level is unusually deep. Explanation in text.

The effect of aspartate on the C-type horizontal cell was, in general, identical with that on the L-type one (Fig. 8*b*). During recovery from the depolarized level to the original state, it is seen in Fig. 8 that the red depolarizing response is more susceptible to aspartate than the blue hyperpolarizing response. In a series of records taken with CR-coupled high amplification (Fig. 8*a*), the red response temporarily reversed its polarity to negativity under the effect of sodium aspartate (see 2, 3, 4, 5 and 6 in Fig. 8*a*). As recovery progressed, an increased depolarizing response was gradually superimposed (7 and 8), until final recovery (9 in Fig. 8*a*).

Almost the same results were obtained with glutamate.

Effects of glycine and GABA

Glycine hyperpolarized the L-type horizontal cell. In the experiment shown in Fig. 9*a*, before application of a chemical, an intense light flash was delivered from a xenon flash lamp through a fibre light guide, in order

to ascertain the ceiling level which the maximally hyperpolarized response could attain. This level indicated by P was, in the later part of the experiment, helpful for monitoring a level of hyperpolarization caused by a chemical. Hyperpolarization brought about by an 8 sec application of glycine reached almost the maximally hyperpolarized level produced by an intense light flash (Fig. 9*a*), or slightly exceeded this level if the chemical was applied continuously.

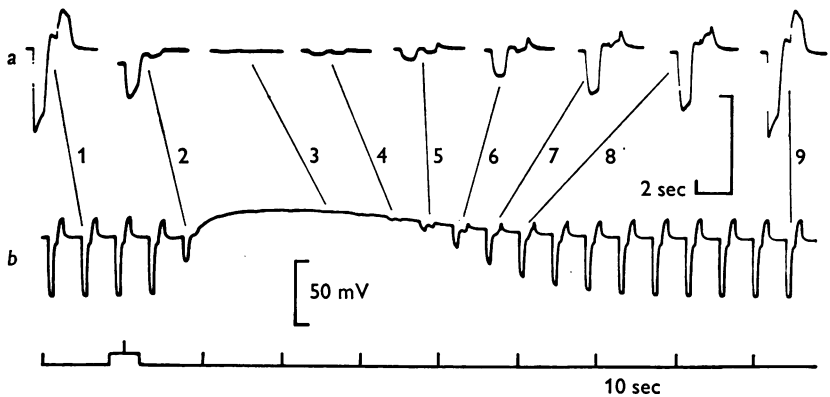

Fig. 8. Difference in susceptibility to aspartate between hyperpolarizing and depolarizing response of C-type horizontal cell. A series of responses shown in *a* are recorded by a CR-coupled amplifier and oscilloscope. Each reponse corresponds, as numbered, to a response in *b* recorded by a direct coupled pen recorder. Time calibration of 2 sec for *a*, and 10 sec for *b*. Voltage calibrations of 50 mV are for both records. Application of aspartate in *b* is indicated by an elevation of the time scale.

The action of glycine on the C-type horizontal cell was, in general identical with that on the L-type one (Fig. 9*b*). The depolarizing responses to red light were more strongly suppressed and showed a longer delay in recovery than the hyperpolarizing response to blue light.

Aspartate rapidly depolarized the cell which had been hyperpolarized by glycine and the cell slowly became repolarized probably because the action of aspartate was removed (Fig. 10*a*). With reverse sequence of chemical applications, a cell once depolarized by aspartate was never rapidly hyperpolarized even by a long-lasting application of glycine (Fig. 10*b*). At the end of the recording, the electrode was withdrawn out of the cell to show an extracellular potential level. These observations indicate that the depolarizing action of aspartate is stronger than the hyperpolarizing action of glycine.

GABA exhibited a hyperpolarizing effect on the horizontal cell.

Effect of ACh

ACh had no action on the horizontal cell, even when the retina was previously eserinized.

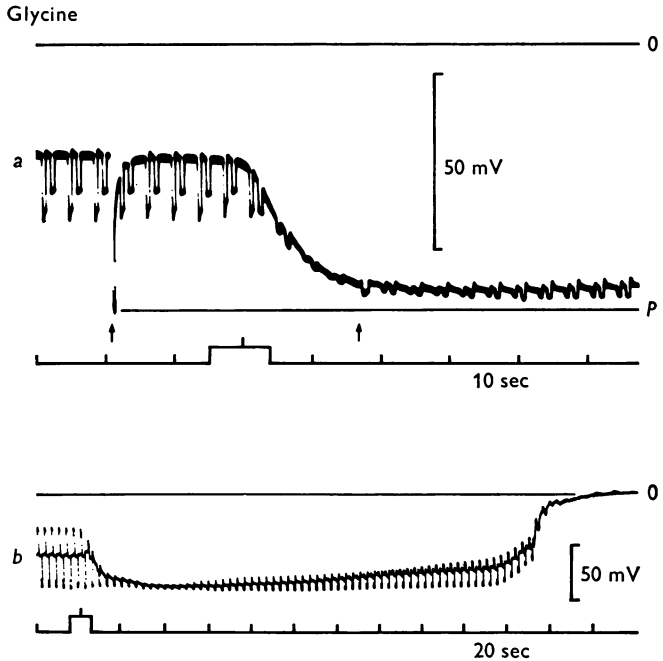

Fig. 9. Effect of glycine on the L-type (*a*) and C-type (*b*) horizontal cells. In record *a*, the horizontal line, *P*, indicates the potential level that a hyperpolarizing S-potential to a strong flash light reached. Timings of flash deliveries are indicated by arrows. At the end of record *b*, the electrode spontaneously slipped out of the cell.

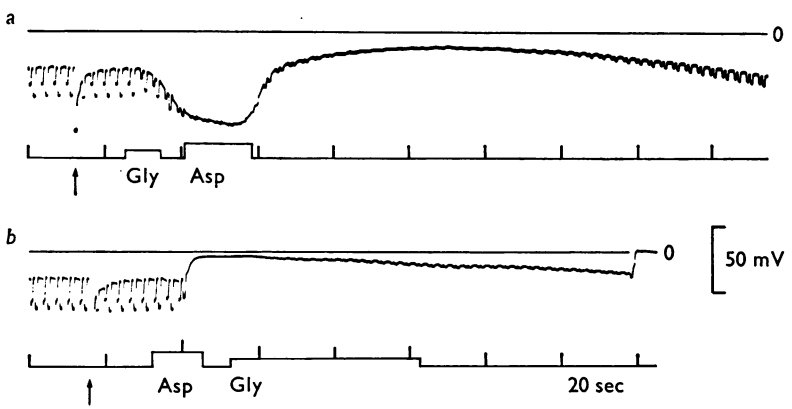

Fig. 10. Successive application of glycine (Gly) and aspartate (Asp) on the L-type horizontal cell. *a*: the cell once hyperpolarized by glycine turned to be depolarized on application of aspartate. *b*: depolarization caused by aspartate was not affected by application of glycine.

DISCUSSION

Mechanism of S-potential generation

Excitatory synapses produce a unique type of hyperpolarization (disfacilitatory hyperpolarization) when a sustained depolarizing bombardment from a presynaptic neurone is removed (Terzuolo, 1959; Llinás, 1964; Tsukahara, Toyama, Kosaka & Udo, 1964; Toyama, Tsukahara & Udo, 1967). The hypothesis proposed by Trifonov (1968) and by Toyoda *et al.* (1969) to explain the mechanism of the hyperpolarizing response of the horizontal cell is in some way similar to this principle of disfacilitatory hyperpolarization: an excitatory transmitter in the receptor terminals is continuously liberated in the dark and ceases to be released in the light, resulting in hyperpolarization of the horizontal cell. The maximum value which the hyperpolarizing S-potential can attain is the difference between the potential level in the dark and a ceiling level probably determined by the equilibrium for some ion or ions. The temporary increase in amplitude of the S-potential seen around midway in recovery from the action of aspartate (Fig. 7) may be explained by assuming that the response in this phase is not completely masked by the action of aspartate, but yet has a sufficient working range between the dark level and the ceiling level to result in an increase in response amplitude.

The mechanism of the depolarizing response of the C-type horizontal cell to longer wave-lengths still remain unknown. There is suggestive evidence, however, that the depolarizing response is relayed indirectly from the receptor: the depolarizing response is more susceptible to aspartate than the hyperpolarizing response (Fig. 8), and the latency of the depolarizing response is consistently longer than that of the hyperpolarizing response (Spekreijse & Norton, 1970).

Are glutamate and aspartate transmitters?

While much has been reported about the putative transmitters in the inner plexiform layer of the vertebrate retina (see the reviews by Lolley, 1969, and by Witkovsky, 1971), no chemical transmitter has been suggested for the region of the outer plexiform layer. On the basis of electrophysiological observations, Trifonov (1968) and Toyoda *et al.* (1969) suggested that the transmitter released from the receptors may be excitatory. As reported here, glutamate and aspartate rapidly and strongly depolarized the horizontal cells. This finding suggests the possibility that these excitant amino acids might be actual transmitters.

The concentration of the amino acids necessary to affect the horizontal cell activity and the e.r.g. components (several mM, see Methods, and also Hanawa & Tateishi, 1970) is not widely different from the concentration

of glutamate that affects cortical neurones when applied iontophoretically (0·5–1·2 mM, cf. Krnjević & Phillis, 1963). These values, however, are about one hundred times higher than that at the neuromuscular junction of the crayfish, where glutamate has been established as a transmitter (Takeuchi & Takeuchi, 1964). One might argue that these amino acids are not transmitters in the central nervous system, including the retina, because such high concentrations must be used. However, this argument is limited and by no means conclusive, since we have no way to measure or estimate the concentrations of chemicals at the very site of the synapses.

Hyperpolarizing action of glycine and GABA

The hyperpolarizing action of glycine (Fig. 9) and GABA is not readily understandable. According to the conventional concept of a chemical synapse, it is hard to accept that more than one kind of transmitter operate at the synapse in the receptor terminals, where only one type of synaptic structure has been revealed by electron microscopic study (Witkovsky & Dowling, 1969). It has been suggested that the transmitter is excitatory (depolarizing) as noted previously, and this interpretation agrees with our results. We have no further information concerning the possible physiological significance of hyperpolarization caused by glycine or by GABA.

Mechanism of b-wave suppression

Sodium aspartate and glutamate are reported to have a potent suppressive effect on the b-wave of the e.r.g. (Furukawa & Hanawa, 1955; Sillman, Ito & Tomita, 1969; Hanawa & Tateishi, 1970; also Fig. 3 in this paper). The results of intracellular recordings from the horizontal cells (Figs. 6–8) clarify the mechanism of the b-wave suppression caused by these chemicals. An amount of the chemical sufficient to fully depolarize the horizontal cells masks the action of the endogenous transmitter released from the receptor terminals. Such concentration of the amino acids is in the range of several mM (see Methods), and roughly agrees with the concentration of these chemical perfusates which could suppress the b-wave of the frog e.r.g. (Hanawa & Tateishi, 1970). Blocking of this type has also been observed in the bipolar cells (M. Murakami & T. Ohtsuka, unpublished data). Therefore, the suppression of the b-wave is apparently due to blocking of the neural pathways at the synapses between the receptors and the following cells. This synaptic blocking inactivates all of the subsequent neurones in the retina including those which produce the b-wave.

The receptor potential was isolated not only by glutamate, but also by glycine (Fig. 4). At the cellular level, however, the mechanism of

suppression of the e.r.g. components is quite different. As shown in Fig. 9, glycine hyperpolarizes the horizontal cells and blocks the synaptic transmission, while glutamate depolarizes them.

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