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EFFECTS OF γ-AMINOBUTYRIC ACID ON ISOLATED CONE PHOTORECEPTORS OF THE TURTLE RETINA

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SUMMARY

1. Isolated cones dissociated from the retina of the freshwater turtle were voltage clamped using a single 'patch' pipette electrode. γ-Aminobutyric acid (GABA) applied ionophoretically to the axon terminal evoked an inward current in cells held at −66 mV when they were recorded with patch pipettes filled with the 'control' pipette solution containing 120 mM-Cl^-.

2. Polarity of the GABA-induced current reversed near 0 mV when examined with the pipette filled with the control pipette solution. The reversal potential depended strongly on both external and intrapipette Cl^- concentrations ($[Cl^-]_o$, and $[Cl^-]_p$). The reversal potential agreed with the equilibrium potential for Cl^- calculated by the Nernst equation with given $[Cl^-]_o$ and $[Cl^-]_p$.

3. The reversal potential was not affected by concentrations of either external Na or K ions.

4. Voltage responses evoked by GABA were hyperpolarizing from its resting level of about −50 mV immediately after the rupture of the patch membrane. The response polarity reversed into depolarization in a few seconds when $[Cl^-]_p$ was > 24 mM, while hyperpolarizing responses persisted when $[Cl^-]_p$ was < 12 mM. Thus, the intracellular Cl^- concentration of undisturbed isolated cones was estimated to be between 12 and 24 mM.

5. Cones were desensitized to GABA (1) in the presence of GABA (> 100 nM) in the medium, or (2) by a prolonged ionophoretic application. The maximum reduction in response amplitude was about 70% in both experiments.

6. Muscimol was as potent as GABA, while β-*p*-chlorophenyl-GABA (baclofen) was ineffective even at 100 μM. GABA was antagonized by bicuculline competitively, and by picrotoxin non-competitively. These observations suggest that turtle cones have $GABA_A$ receptors which associate with chloride channels.

7. The present results suggest that GABA, presumably released continuously from monophasic horizontal cells in the dark, would exert a tonic hyperpolarization in red-sensitive and green-sensitive cones. Suppression by light of tonic GABA release would depolarize these types of cones by disinhibition. Disinhibitory depolarization in cones may contribute to the centre surround antagonism in retinal neurones, and to the biphasic colour responses recorded in a subtype of horizontal cells.

INTRODUCTION

It is widely believed that the horizontal cell in the retina plays an important role in formation of the receptive field surround (Werblin & Dowling, 1969; Baylor & Fuortes, 1970; Kaneko, 1970) and of colour opponent responses (Fuortes, Schwartz & Simon, 1973; Fuortes & Simon, 1974; Stell & Lightfoot, 1975; Stell, Lightfoot, Wheeler & Leeper, 1975). These suggestions were made by comparing the receptive field sizes of various types of distal retinal neurones, such as photoreceptors, bipolar cells and horizontal cells, or by comparing spectral responses of several subtypes of horizontal cells. Horizontal cells are post-synaptic to photoreceptors and send their signals back to photoreceptors through a negative feed-back pathway. This feed-back connexion was first demonstrated by Baylor, Fuortes & O'Bryan (1971) in their observation that polarization of a horizontal cell in the turtle retina by an extrinsic current evoked electrical responses of the opposite polarity in nearby cones. Furthermore, O'Bryan (1973) and Fuortes & Simon (1974) found that the effect of feed-back in the turtle is specific among cones and horizontal cells of particular spectral sensitivity.

γ-Aminobutyric acid (GABA) has been suggested as the neurotransmitter of the monophasic subtype of horizontal cells. This type is characterized by its hyperpolarizing response to monochromatic light flashes of all visible wavelengths (Simon, 1973; Saito, Miller & Tomita, 1974). It has been shown in fish and toad that monophasic horizontal cells synthesize GABA (Lam, 1972; Lam, Su, Swain, Marc, Brandon & Wu, 1979), accumulate exogenous GABA through a high affinity uptake mechanism (Lam & Steinman, 1971; Marc, Stell, Bok & Lam, 1978; Schwartz 1982) and release stored GABA when exposed to high $[K^+]_o$ (Schwartz 1982; Ayoub & Lam, 1984) or to L-glutamate (Miller & Schwartz, 1983; Ayoub & Lam, 1984). GABA was shown to hyperpolarize the dark membrane potential of green-sensitive carp cones (Murakami, Shimoda, Nakatani, Miyachi & Watanabe, 1982*a*). Contribution of the GABAergic synapse to colour opponent responses of biphasic and triphasic horizontal cells was suggested by a demonstration that application of GABA agonists or antagonists in the fish retina affects chromatic responses of those horizontal cells (Murakami, Shimoda, Nakatani, Miyachi & Watanabe, 1982*b*; Toyoda & Fujimoto, 1983).

Based on these suggestions that the monophasic horizontal cell is GABAergic, Tachibana & Kaneko (1984) examined sensitivity to GABA of isolated photoreceptors dissociated from the turtle retina. They found that red-sensitive and green-sensitive cones were highly sensitive to GABA, and that the sensitivity was localized at the axon terminals. GABA-sensitivity in blue-sensitive cones and rods was very low. In this work, ionophoretically applied GABA induced an inward current when the membrane potential was held at -60 mV, and the reversal potential of GABA-induced responses was near 0 mV. Since GABA is thought to hyperpolarize photoreceptors, the observed polarity of GABA-induced current was opposite to what is expected for the action of GABA on cones. The present study was made to clarify the nature of GABA-induced responses in the cone photoreceptor of turtle and to correlate them with the function of horizontal cells.

METHODS

Eyes were excised from the dark-adapted freshwater turtle, *Geoclemys reevesii* (*ca.* 20 cm carapace length) after decapitation and pithing. The eyes were opened along the ora serrata, and incubated at 24 °C for approximately 30 min in a solution containing 6 u./ml papain (Worthington, Biochemical Corp, Freehold, NJ, U.S.A.) as reported previously (Tachibana & Kaneko, 1984). The solution was saturated with oxygen and gently stirred. This initial incubation loosened attachment of the retina to the pigment epithelium. The isolated retina was cut into several pieces, and incubated again in a fresh incubating medium of identical composition for 20–40 min. Duration of incubation was adjusted according to the activity of the enzyme. At the end of incubation, small pieces of the retina were rinsed several times in enzyme-free medium, and triturated with a large-tipped pipette (*ca.* 1·5 mm i.d.). Cells were plated in a culture dish and incubated for 2–3 h until they attached to the bottom of the dish made of a concanavalin A-coated cover glass.

Identification of cells

For recordings, the culture dish was mounted on the stage of an inverted microscope with phase-contrast optics (Nikon, type TMD, Tokyo, Japan). Photoreceptors were unequivocally identified by their morphology (Ohtsuka, 1984, 1985) which was maintained after dissociation (Pl. 1). Since single cones containing red oil droplet (red-sensitive cone) have high sensitivity to GABA (Tachibana & Kaneko, 1984), recordings were made mainly from this type of cone in the present study. A few cells of other types were examined for comparison, but the results were essentially similar.

Recording procedures

Isolated cones were recorded by using 'patch' pipettes in the whole-cell clamp configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The control pipette solution contained (in mM): 120, KCl; 5, EGTA; 10, HEPES, and pH was adjusted to 7·3 by adding KOH (final concentration of KOH was approximately 18 mM). Low Cl^- pipette solution was made by molar to molar substitution of glutamate (glutamic acid–KOH) for Cl^-. The control pipette solution contained higher Cl^- concentration than intracellular Cl^- concentration ($[Cl^-]_i$) reported for frog rods (Somlyo & Walz, 1985). We used high Cl^- concentration, since it gave large GABA-induced currents making various analyses easier. Ions and other small molecules diffused freely from the inside of the recording pipette to the cell interior, which was confirmed from the two observations. First, a rapid (< 30 s) diffusion of a fluorescent dye, Lucifer yellow, was seen under a fluorescence microscope after rupturing the patch membrane. Secondly, > 2 min after rupture of the patch membrane, the reversal potential of GABA-induced current was very close to the equilibrium potential for Cl^- (E_{Cl}) calculated with Cl^- concentrations of superfusate ($[Cl^-]_o$) and of the pipette solution ($[Cl^-]_p$) (see Results). In most experiments GABA-induced currents were measured after this period. During recording, cells were superfused by a solution containing (in mM): NaCl, 79; KCl, 10; $CaCl_2$, 2·5; $MgCl_2$, 1; glucose, 16; HEPES, 2; choline Cl, 37 (maintained at 15 °C and pH 7·4). Low chloride extracellular medium was prepared by molar to molar substitution of methanesulphonate for Cl^-.

Correction of the liquid junction potential

The liquid junction potentials between the bath and the pipette solutions were measured with a 3 M-KCl-filled micro-electrode, by assuming that the junction potential at the tip of a KCl-filled micro-electrode in these solutions is negligible (Hagiwara & Ohmori, 1982). Patch pipettes were filled each with a solution of different $[Cl^-]_p$ used in the present experiments. Each of these pipettes was placed in the standard superfusate, and potentials were measured first by placing the KCl-filled micro-electrode in the bath, and then by moving it to make contact with the solution inside the patch pipette. The potential difference between the bath and the patch pipette was taken as the liquid junction potential at the pipette tip. It was approximately 6 mV at $[Cl^-]_p$ 120 mM, 10 mV at 60 mM, 14 mV at 36 mM, 15 mV at 24 mM, and 17 mV at 12 mM, all being negative inside the pipette. The junction potential is present when the pipette is in the bath, but is thought to be minimal between the pipette and the cytoplasm after the intracellular ionic composition becomes close to that of the pipette solution by diffusion of ions through the ruptured membrane patch.

Since these potential levels outside the bath were taken as the reference, the real membrane potentials were more negative than the observed values by these amounts.

The junction potential of the ground electrode was similarly measured with a 3 M-KCl-filled micro-electrode when the superfusate containing various $[Cl^-]_o$ was introduced to the bath. The largest (about 5 mV) junction potential was obtained when 90% of Cl^- in the superfusate was replaced with methanesulphonate. The membrane potentials described in the present paper are values corrected for these junction potentials by calculation.

Application of test substances

Pharmacological agents used in this study were GABA, bicuculline methochloride, picrotoxin, muscimol, commercially supplied from Sigma (St. Louis, MO, U.S.A.). β-*p*-Chlorophenyl-GABA (baclofen) was a generous gift of Ciba-Geigy (Japan) Ltd. The agents were dissolved freshly before use. GABA was applied either by ionophoresis or by pressure ejection. For ionophoresis, 1 M-GABA (pH 4·0) was filled in a glass micropipette (tip diameter about 0·1 μm, resistance *ca.* 300 MΩ), which was positioned close to the cone pedicle, the site of the highest GABA sensitivity. A small amount of steady current of the opposite polarity (adjusted for each pipette, usually 3–5 nA) prevented GABA leakage from the pipette tip. GABA was applied by passing brief current pulses (duration 1–50 ms, intensity 1–50 nA) from a current source. For pressure ejection, GABA (100 nM–10 μM) dissolved in the superfusate was filled in a 20 μm-tip pipette which was placed approximately 20 μm away from the cell. A 3 s pulse of 0·4 kg/cm² was applied to the pipette to eject GABA (for details, see Ishida, Kaneko & Tachibana, 1984; Tachibana, 1985).

Under these conditions, it was possible to hold a cell for nearly half an hour without serious changes in morphology. However, membrane currents deteriorated gradually, particularly when the cell was strongly polarized or when the cell was perfused with a high concentration of pharmacological agents. A total of 125 cells were studied. Data presented in this report includes at least three cells, unless otherwise mentioned, for each experiment with identical results. Other details of recording methods and data processing have been described elsewhere (Kaneko & Tachibana, 1985).

RESULTS

GABA current evoked in isolated cones

The resting membrane potential of isolated cone photoreceptors was usually between −30 mV and −45 mV (−37·6 ± 7·6 mV, mean ± S.D.; $n = 92$, number of cells examined). In cells voltage clamped at −66 mV, ionophoretic application of GABA at the cone pedicle evoked an inward current of large amplitude usually exceeding 100 pA. As illustrated in Fig. 1*A*, the amplitude of the GABA-induced inward current decreased by membrane depolarization, and was almost nullified at approximately 0 mV. Further depolarization reversed the polarity of the GABA response into outward direction, and the amplitude of the reversed current increased by further depolarization. The reversal potential was −4 mV (−1·3 ± 3·6 mV, $n = 40$), and was almost identical independent of the cell type or the location of GABA application. The relationship between the GABA-induced current and the membrane potential was nearly linear with a slope conductance of about 1·9 nS near the reversal potential (Fig. 1*B*), but in some preparations showed a weak outward rectification (see below). These results indicate that GABA increased the membrane conductance to a species (or species) of ions, the equilibrium potential of which was near 0 mV.

Dependence of the reversal potential on Cl^- concentrations

If one assumes that a single species of ions go through the GABA-induced channels, Cl^- is the most likely candidate, since the estimated Cl^- equilibrium potential (E_{Cl})

is close to the observed reversal potential. On this assumption we next studied the effects of $[Cl^-]_o$ on the reversal potential of the GABA-induced responses (Fig. 2). In the experiment of Fig. 2*A*, a cell was first recorded in the standard medium ($[Cl^-]_o$ = 133 mM) and the relationship between the GABA-induced current and the

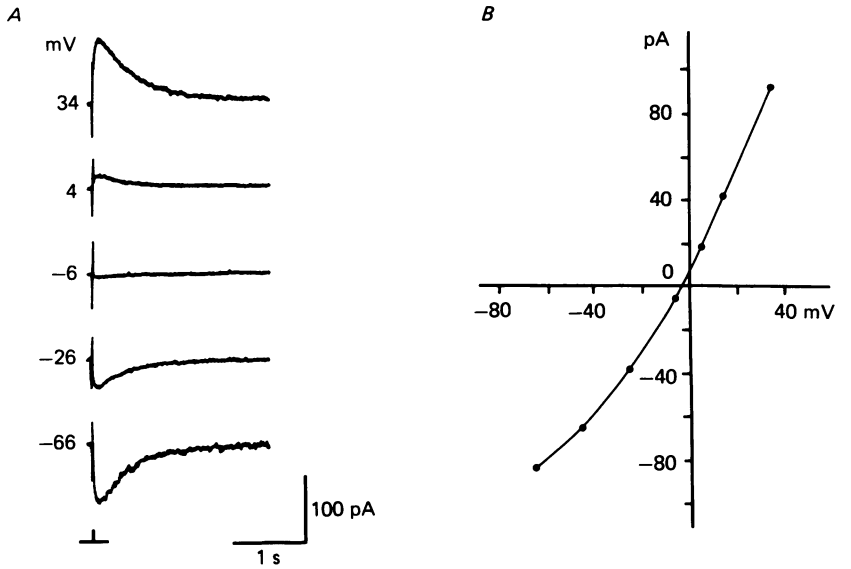

Fig. 1. Reversal potential of GABA-induced currents recorded from a cone with red oil droplet (red-sensitive cone). Whole-cell recording using a patch pipette filled with the 'control' solution ($[Cl^-]_p$ = 120 mM). *A*, the cell was voltage clamped at potentials indicated to the left of each trace, and GABA was applied ionophoretically to the cone pedicle from a fine glass pipette. The timing of ionophoretic current (intensity +50 nA, duration 10 ms) is indicated at the bottom. Brake current of −18 nA was continuously passed to prevent leakage of GABA from the pipette. Current traces were shifted vertically by an arbitrary amount. *B*, current–voltage relationship of GABA-induced responses measured at their peaks.

membrane potential was examined (●). Next, similar measurements were repeated by superfusing the cell in low Cl^- media ($[Cl^-]_o$ = 45 mM, ▲; $[Cl^-]_o$ = 7 mM, ■). As $[Cl^-]_o$ was decreased, the reversal potential shifted to more positive values and the slope of the current–voltage relationship became smaller. The amount of GABA-induced conductance was between 1 and 2 nS (1·7 ± 0·5 nS, n = 15) in the standard medium in response to the saturating dose of GABA, but was smaller in low Cl^- media. It was approximately 66 % of maximum at $[Cl^-]_o$ 95 mM, 47 % at $[Cl^-]_o$ 70 mM and < 5 % in $[Cl^-]_o$ 7 mM. Fig. 2*B* illustrates the relationship between the reversal potential of GABA-induced response and $[Cl^-]_o$. The observed reversal potentials were very close to the value estimated from various $[Cl^-]_o$ and $[Cl^-]_p$ (120 mM) by the Nernst equation.

The reversal potential of the GABA-induced current depended also on $[Cl^-]_i$ (Fig. 3). In these experiments each cell was superfused with the standard medium ($[Cl^-]_o$ = 133 mM) and voltage clamped using a patch pipette filled with a solution of different $[Cl^-]_p$. The pipette solution diffused to the cell interior and changed $[Cl^-]_i$.

Fig. 3*A* illustrates the relationship between the GABA-induced current and the membrane potential recorded in three cells using different $[Cl^-]_p$. As $[Cl^-]_p$ was decreased, the reversal potential shifted to more negative values and the slope of the current–voltage relationship (GABA-induced conductance) became smaller

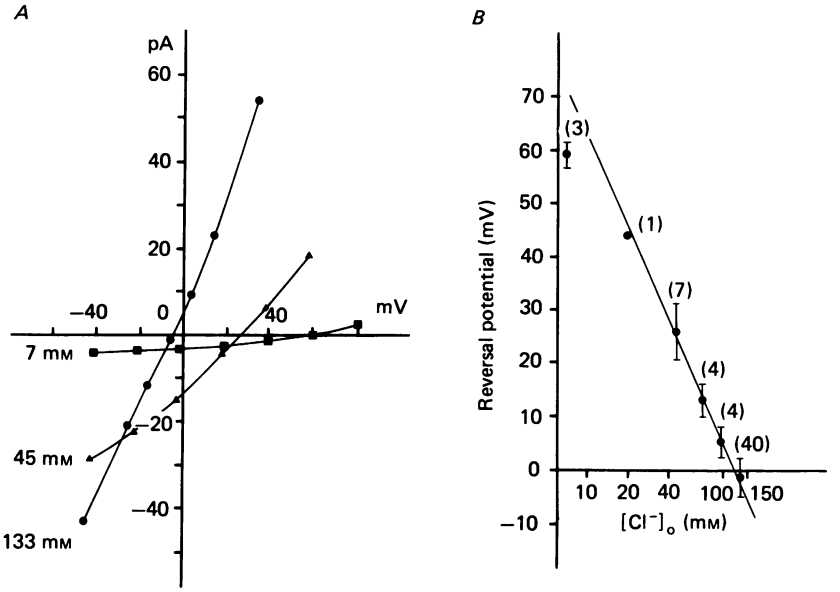

Fig. 2. Effects of $[Cl^-]_o$ on the GABA-induced current in cones with red oil droplet. *A*, current–voltage relationship of GABA-induced responses measured at their peaks in a cone superfused with three different $[Cl^-]_o$: 133 mM (●), 45 mM (▲), and 7 mM (■) applied in sequence. Low $[Cl^-]_o$ was prepared by molar to molar substitution of methanesulphonate for Cl^-. GABA was applied ionophoretically to the cone pedicle by passing 40 nA pulses of 10 ms duration (near the saturating dose). Ten-times dose was given when recorded in $[Cl^-]_o$ 7 mM. The GABA-induced conductance of this cell (the slope conductance near the reversal potentials) was approximately 1·1 nS in $[Cl^-]_o$ 133 mM, 0·4 nS in $[Cl^-]_o$ 45 mM and 0·1 nS in $[Cl^-]_o$ 7 mM. *B*, reversal potentials of the GABA-induced current measured in cells superfused by a solution containing various $[Cl^-]_o$. Average (●) and ±standard deviation (bars) of reversal potentials. Numbers in parentheses indicate the sample size. The line represents Cl^- equilibrium potential calculated from various $[Cl^-]_o$ and $[Cl^-]_p$ (120 mM) by the Nernst equation.

(1·7 ± 0·5 nS at 120 mM, $n = 15$; 1·2 ± 0·4 nS at 36 mM, $n = 4$; 1·1 ± 0·6 nS at 24 mM, $n = 5$; 0·5 nS at 12 mM, $n = 2$). Fig. 3*B* illustrates the relationship between the reversal potential and $[Cl^-]_p$. The observed reversal potentials were very close to the value estimated from various $[Cl^-]_p$ in the patch pipette and $[Cl^-]_o$ by the Nernst equation, indicating that $[Cl^-]_i$ was nearly identical to $[Cl^-]_p$ and that the GABA-induced response strongly depends also on $[Cl^-]_i$.

Either in the Na^+-free medium (replaced with choline, three cells) or K^+-rich medium ($[K^+]_o = 40$ mM, five cells), neither the amplitude nor the reversal potential of the GABA-induced responses changed significantly. From these observations, we concluded that GABA increased the membrane conductance selectively to Cl^- in isolated turtle cones.

The wave forms of the GABA-induced responses at various membrane potentials were very similar to one another (Fig. 1*A*), but close examination revealed that the response evoked at hyperpolarized potentials had faster rise and fall than those evoked at depolarizing potentials. However, since such changes in wave form were small, they are not discussed further in detail.

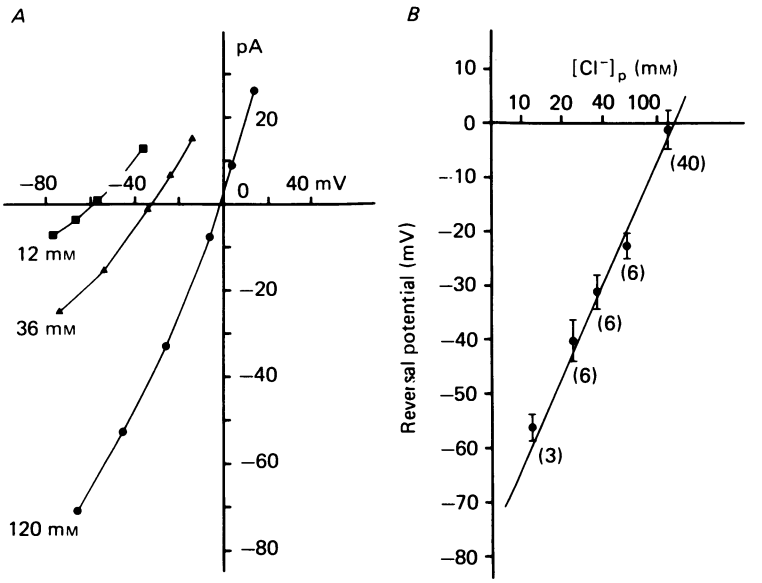

Fig. 3. Effects of $[Cl^-]_p$ on the GABA-induced currents in cones with red oil droplet. *A*, current–voltage relationship of GABA-induced responses measured at their peaks in three cones recorded with patch pipette each filled with three different $[Cl^-]_p$: 120 mM (●), 36 mM (▲), and 12 mM (■). Low $[Cl^-]_p$ was prepared by molar to molar substitution of glutamate for Cl^-. GABA was applied ionophoretically to the cone pedicle by passing 50 nA pulses of 20 ms duration (near the saturating dose). 50 nA pulses of 30 ms duration were given when recorded with $[Cl^-]_p$ 12 mM. GABA-induced conductances (the slope conductance near the reversal potentials) were approximately 1·5 nS in $[Cl^-]_p$ 120 mM, 0·8 nS in 36 mM and 0·5 nS in 12 mM. *B*, reversal potentials measured in cells recorded with patch pipettes filled with a solution of various $[Cl^-]_p$. Average (●) and ±standard deviation (bars) of cells (sample number is indicated in parenthesis). The line represents the Cl^- equilibrium potential calculated from $[Cl^-]_o$ (133 mM) and various $[Cl^-]_p$ by the Nernst equation. The junction potential was corrected by calculation (detail in Methods).

Voltage responses recorded immediately after the rupture of the patch membrane

The above experiments have shown that the reversal potential of GABA-induced current strongly depended on Cl^- concentrations on either side of the membrane. Since GABA-induced responses were examined after $[Cl^-]_i$ became close to $[Cl^-]_p$, GABA produced an inward current when the patch pipette was filled with a solution of high $[Cl^-]_p$. If we record voltage responses under these conditions, GABA would depolarize cones, opposite to the expectation that it hyperpolarizes cones. To identify the polarity of GABA-induced responses of unruptured cells, it is important to know their $[Cl^-]_i$. In the following experiments, we measured the voltage responses to

GABA, applied to the axon terminal, immediately after the rupture of patch membrane to estimate $[Cl^-]_i$ of unruptured cones.

In the experiment of Fig. 4*A*, gigaohm seal was established with a recording pipette filled with $[Cl^-]_p$ 36 mM. The patch membrane was ruptured at the instant

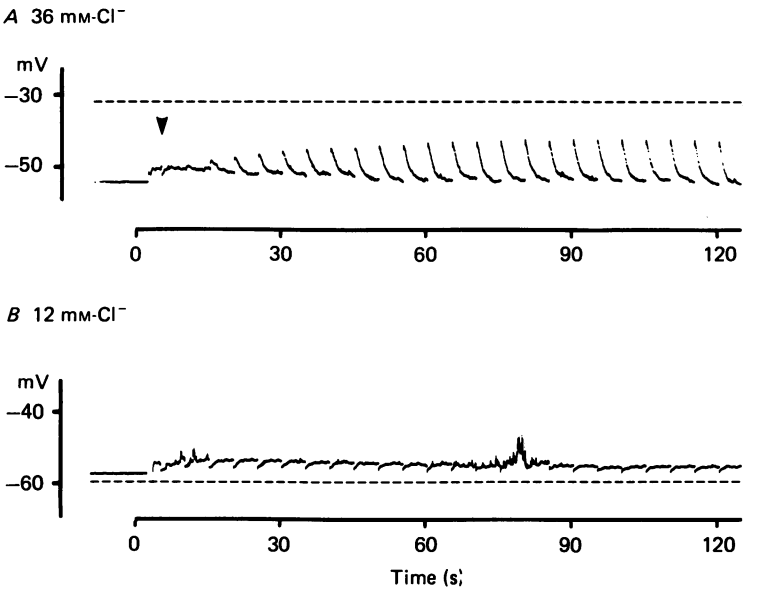

Fig. 4. GABA-induced voltage responses recorded from cones containing red oil droplet. First, gigaohm seal was established between the pipette tip and the cell surface (at ellipsoid) while applying a holding potential of −54 mV (*A*) or −57 mV (*B*). The patch membrane was ruptured at time 0, and the recording system was switched from voltage-clamp into current-clamp configuration (indicated by an upward shift in the voltage trace). *A*, recording pipette was filled with a low Cl^- solution ($[Cl^-]_p$ = 36 mM by substitution of glutamate for Cl^-). GABA was applied to the cone pedicle repetitively once every 5 s by passing ionophoretic current pulses (intensity +35 nA, duration 30 ms, brake current −5 nA) to a GABA-filled micropipette. Note that the polarity of the initial response after the rupture of the patch membrane (arrowhead) is hyperpolarizing, and it reverses at the second and subsequent applications of GABA. Interrupted line indicates the reversal potential of GABA-induced current (−32 mV) measured under the voltage-clamp condition > 3 min after the rupture of the patch membrane. *B*, similar recording from another cone made with a pipette filled with $[Cl^-]_p$ = 12 mM. GABA was applied repetitively every 5 s by ionophoresis (intensity +50 nA, duration 20 ms, brake current −8 nA). Note that all responses are hyperpolarizing. Interrupted line indicates the reversal potential of GABA-induced current (−59 mV) measured under the voltage-clamp condition > 3 min after the rupture of the patch membrane.

indicated as time 0. Within a few seconds, the recording system was switched from the voltage-clamp (holding potential, −54 mV) to current-clamp configuration, as indicated by an upward shift, by a few mV, of the voltage trace (resting potential −51 mV). The resting membrane potential gradually hyperpolarized with time and became steady in about 1 min. The amount of resting potential during this period was within 4 mV.

(The liquid junction potential at the tip of the patch pipette filled with $[Cl^-]_p$ 36 mM

was 14 mV in the standard superfusate (see Methods), which is thought to disappear after $[Cl^-]_i = [Cl^-]_p$. A change in the resting potential may be a reflexion of the process with which the junction potential between the pipette solution and the cytoplasm disappeared. Since the change in resting potential was small, it may be either that the junction potential between the patch pipette and the cytoplasm was small, or that the junction potential changed very rapidly within the initial few seconds after the rupture of the patch membrane.)

In response to the initial application of GABA after the membrane rupture, the cell showed a small hyperpolarizing response (arrow head), indicating that the reversal potential was more negative than −50 mV at this moment. The polarity of the GABA response reversed from hyperpolarization to depolarization at the second and subsequent applications of GABA, and the amplitude of the depolarizing responses increased gradually with time until it reached a steady level at about 1 min after the rupture. At the steady state, the reversal potential measured under voltage-clamp condition was −32 mV, as shown by the broken line. The similar polarity reversal of GABA-induced response was observed at any $[Cl^-]_p$ higher than 24 mM.

When similar experiments were made using a recording pipette filled with $[Cl^-]_p$ 12 mM, the polarity of GABA-induced voltage changes remained hyperpolarizing even 2 min after the rupture of the patch membrane (Fig. 4*B*). The reversal potential measured 3 min after the rupture was −59 mV (interrupted line). These observations strongly suggested that unruptured isolated cones have $[Cl^-]_i$ between 24 and 12 mM. If one assumes $[Cl^-]_i$ to be 20 mM as an approximation, E_{Cl} of these cells could be estimated as −47 mV in reference to the experiment of Fig. 3*B*.

The time course of polarity reversal of GABA-induced responses depended on the position of the recording pipette. Records shown in Fig. 4*A* and *B* were taken by positioning the pipette at the ellipsoid region of isolated cones. Similar changes, but with much faster time course, were seen when the recording pipette was located either at the cell body or at the paraboloid region. Difference in time course is probably due to the difference in distance from the recording pipette to the cone pedicle and to an intracellular diffusion barrier which seems to be present between ellipsoid and paraboloid.

Desensitization

Since retinal horizontal cells *in situ* show graded and sustained responses, release of GABA from horizontal cells is likely to be tonic. It is therefore important to ask whether sensitivity to GABA of cones changes during prolonged application of agonists. We obtained signs of desensitization from the following two kinds of experiments.

Response amplitudes of repetitively applied GABA were suppressed when a low concentration of GABA was bath-applied (Fig. 5). 1 μM-bath-applied GABA evoked about 20 pA steady inward current, and suppressed the response amplitude to ionophoretically applied GABA from *ca.* 170 pA to *ca.* 50 pA (by about 72%; Fig. 5*A* and *C*). The suppression was not due to non-linear compression of response amplitude along the sigmoidal dose–response relation (which will be shown later in Fig. 6), since the peak response level measured from the base line (transient response

amplitude plus 20 pA steady current) was smaller in 1 μM-GABA than in the control medium, as seen in Fig. 5*A*. Furthermore, the reduction in response amplitudes during desensitization was not due to the decrease of driving force for Cl^-, since the reversal potential of the GABA response did not change significantly (not shown).

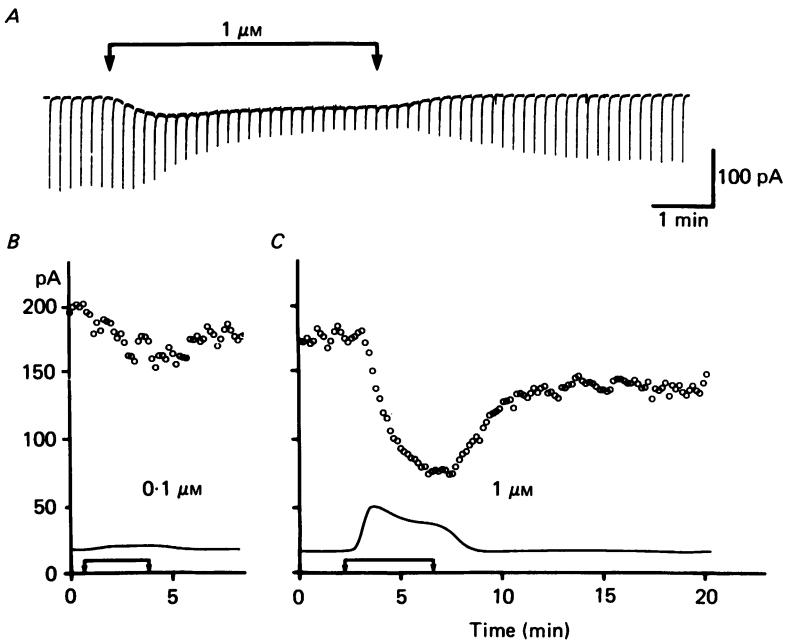

Fig. 5. Desensitization of GABA response. *A*, a continuous record from a cone containing red oil droplet, voltage clamped at −66 mV with a pipette filled with $[Cl^-]_p$ 120 mM solution. Repetitive (0·1/s) application of GABA to the cone pedicle by ionophoretic pulses (intensity +40 nA, duration 5 ms, brake current −5 nA). Addition of 1 μM-GABA to the superfusate (between arrows) induced a sustained inward current, and reduced the amplitude of each GABA response. *B*, effect of 100 nM-GABA bath-applied to the superfusate. Open circles (○) indicates the peak amplitude of each GABA-induced current measured from the base line (the current level when no GABA was added to the bath). Continuous line indicates the level of sustained current measured from zero level. The small amount of steady current (*ca.* 20 pA) recorded in the absence of GABA indicates the background membrane current recorded at −66 mV. *C*, similar recording from the same cell as in *A* and *B* during application of 1 μM-GABA.

Suppression was 63±15% in average ($n = 8$) at 1 μM-GABA in the bath. Desensitization was evident even at 100 nM (Fig. 5*B*). At this concentration, the amplitude of the steady inward current was small (< 5 pA), but suppression of the response to ionophoretically applied GABA was by about 17% (10±6%, three experiments). Suppression at 300 nM-GABA was 28±6% in average of seven cells (see also Fig. 6). Desensitization recovered rapidly and nearly completely after GABA was washed out from the bath. Threshold GABA dose for desensitized cones was nearly equal to that obtained under the control condition, but the maximum response amplitude was reduced in proportion to the amount of suppression to a test dose as mentioned above (Fig. 6).

The second type of desensitization experiment was made to observe the time course of desensitization at its initial phase. For this purpose GABA was applied by an ionophoretic pulse of long duration. A sustained response was obtained to a small dose of GABA which evoked about 10 % of the maximum response (Fig. 7*A*). Random fluctuation of membrane current was seen to increase during this sustained response.

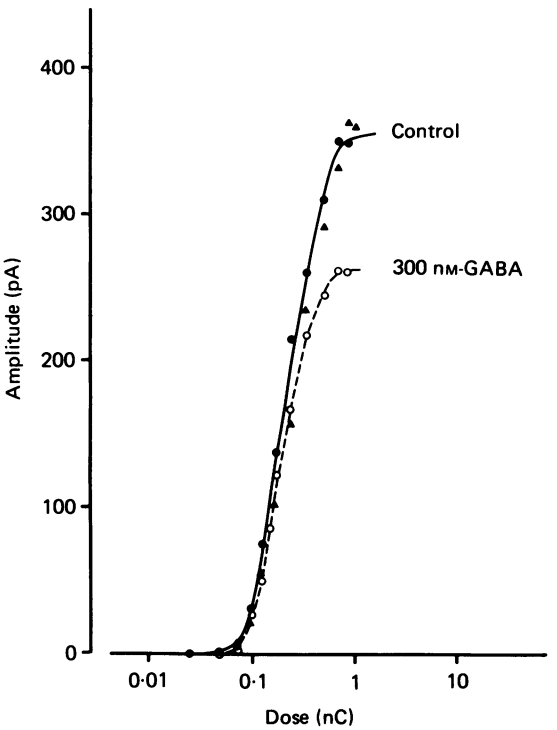

Fig. 6. Dose–response curve of GABA examined in a cone containing red oil droplet, before (●) and during (○) application of 300 nM-GABA in the superfusate (which produced 20 pA steady current) and after washout (▲). Test pulses of GABA were applied to the cone pedicle ionophoretically. The dose (in nC) is the product of the intensity (varied from 5 to 35 nA) and duration (varied from 5 to 45 ms) of ionophoretic current. Brake current, −5 nA. Peak response amplitude is plotted on the ordinate.

At higher doses of GABA (which produced > 20 % of the maximum response), the response showed a relaxation during the 20 s pulse, indicating a desensitization. The higher the dose of GABA, the more strongly and the more rapidly were cones desensitized. The maximal suppression observed in five experiments of this type was about 60 %. These observations suggest that GABA sensitivity of red-sensitive and green-sensitive cones in *in situ* retina may be partially desensitized, but not abolished during the tonic exposure to endogenous GABA released from horizontal cells (see Discussion).

Dose–response relationship

Amplitudes of GABA-induced responses showed a sigmoidal relationship to the dose of GABA (Fig. 8*A*) and saturated within about 2 log unit range. The same data

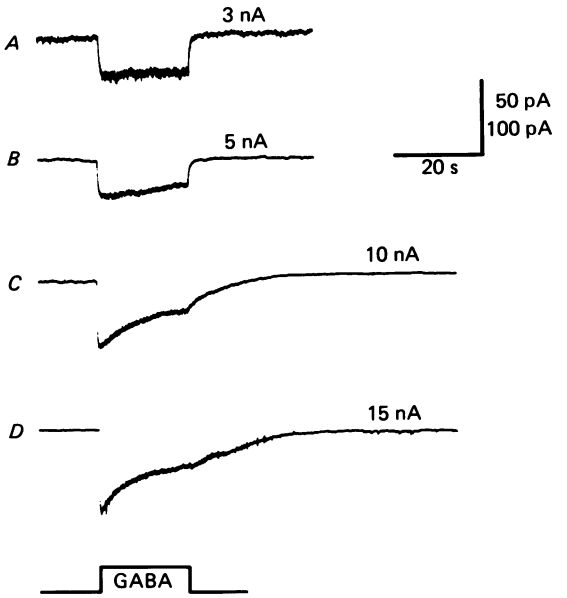

Fig. 7. Response of a cone containing red oil droplet to a 20 s steady pulse of ionophoresis. Holding potential −66 mV, recorded with a pipette filled with $[Cl^-]_p$ 120 mM solution. The GABA pipette was positioned at the cone pedicle. Brake current, −6 nA. Recording gain for trace *A* is twice as large as that for traces *B* to *D*.

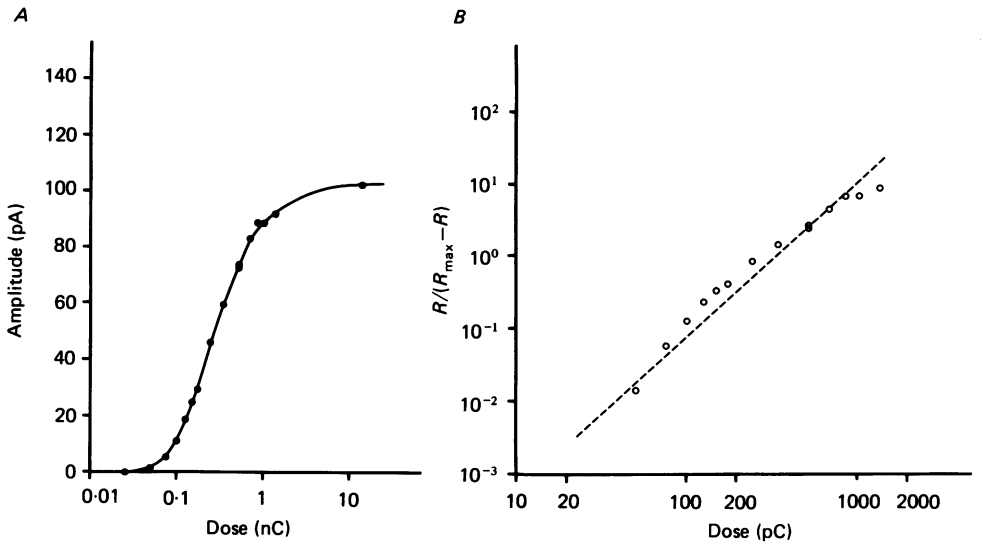

Fig. 8. Peak amplitude of GABA-induced current in a cone containing red oil droplet, plotted on a semilogarithmic coordinate (*A*) and on a Hill coordinate (*B*). Holding potential, −66 mV. Test pulses of GABA were applied to the cone pedicle ionophoretically. The dose is the product of the intensity (varied from 5 to 35 nA) and duration (varied from 5 to 35 ms) of ionophoretic current. Brake current, −5 nA. Continuous line in *A* was drawn by eye, and the interrupted line in *B* was calculated by the least squares method. Hill coefficient of this cell is 1·9.

were used to examine the cooperativity of GABA receptors by estimating the Hill coefficient. Ratios of the response amplitude (R) to the difference between the saturating response (R_{max}) and the amplitude to the given dose (D), $R/(R_{max}-R)$, were plotted against the dose on the double logarithmic scale (Fig. 8*B*). The relationship was linear having a Hill coefficient of approximately 2. Similar values were obtained in other cones. These results indicate that at least two GABA molecules interact with a single molecule of GABA receptor as reported in other preparations, such as crayfish neuromuscular junctions (Takeuchi & Takeuchi, 1969, 1975).

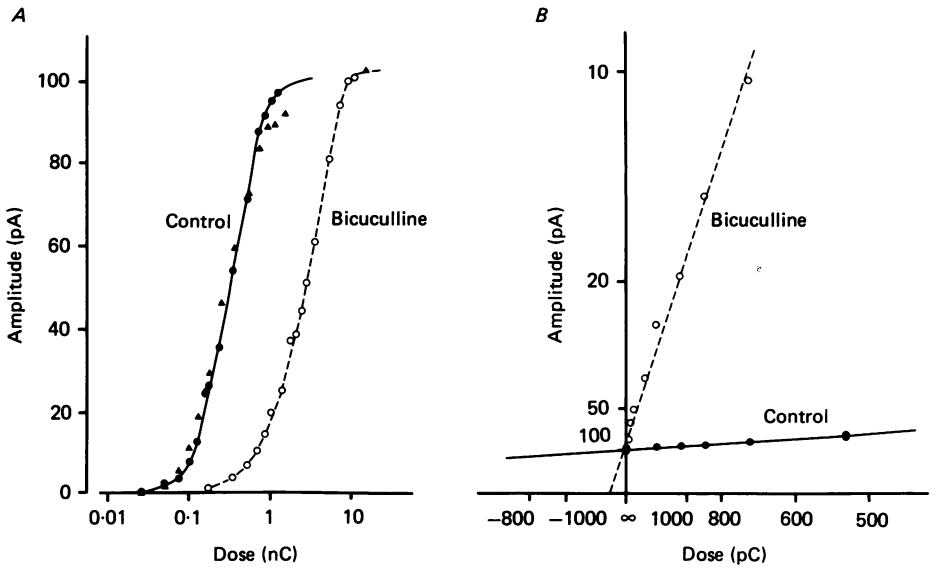

Fig. 9. Effect of bicuculline on the dose–response relationship of GABA in a cone containing red oil droplet. The cone was voltage clamped at −66 mV with a patch pipette filled with $[Cl^-]_p$ 120 mM solution. Test pulses of GABA were applied to the cone pedicle ionophoretically. The dose is the product of the intensity (varied from 5 to 35 nA) and duration (varied from 5 to 300 ms) of ionophoretic current. Brake current −4 nA. *A*, peak amplitude of GABA-induced current plotted on a semilogarithmic coordinate. Control (●), in the presence of 10 μM-bicuculline (○), after wash-out of bicuculline (▲). *B*, reciprocal of peak amplitude is plotted against reciprocal of the square of dose (by assuming cooperativity of GABA to be 2). Lines are fitted by the least squares method. K_D for GABA is 310 pC (no bicuculline) and 1900 pC in 10 μM-bicuculline. I_{max} is 98 pA (no bicuculline) and 83 pA (in 10 μM-bicuculline).

Properties of GABA receptors

GABA receptors are differentiated into two subtypes and designated $GABA_A$ and $GABA_B$, by their selective activation by GABA agonist, muscimol and baclofen (Bowery, Hill, Hudson, Doble, Middlemiss, Shaw & Turnbull, 1980; Hill & Bowery, 1981). $GABA_A$ receptors are sensitive to an antagonist, bicuculline (Curtis, Duggan, Felix & Johnston, 1971), and are related to the activation of Cl^- channels, while $GABA_B$ receptors are bicuculline insensitive, and are coupled with either Ca (Dunlap, 1981) or K channels (Gähwiler & Brown, 1985; Newberry & Nicoll, 1985). The type of GABA receptors in isolated cones was identified by examining the aforementioned properties of GABA receptors.

Effects of GABA antagonists. GABA-induced responses in isolated cones were suppressed either by bicuculline (Fig. 9) or by picrotoxin (Fig. 10). The response amplitudes decreased from the control value (●) in the presence of 10 μM antagonists (○). However, the mechanism of suppression was different. In the presence of 10 μM-bicuculline, the dose–response curve of GABA was shifted towards the direction of higher dose along the abscissa (Fig. 9*A*). Namely, the threshold dose and the saturating dose of GABA increased, but the maximum response amplitude (R_{max}) remained almost the same. The effect of bicuculline was completely reversible (▲).

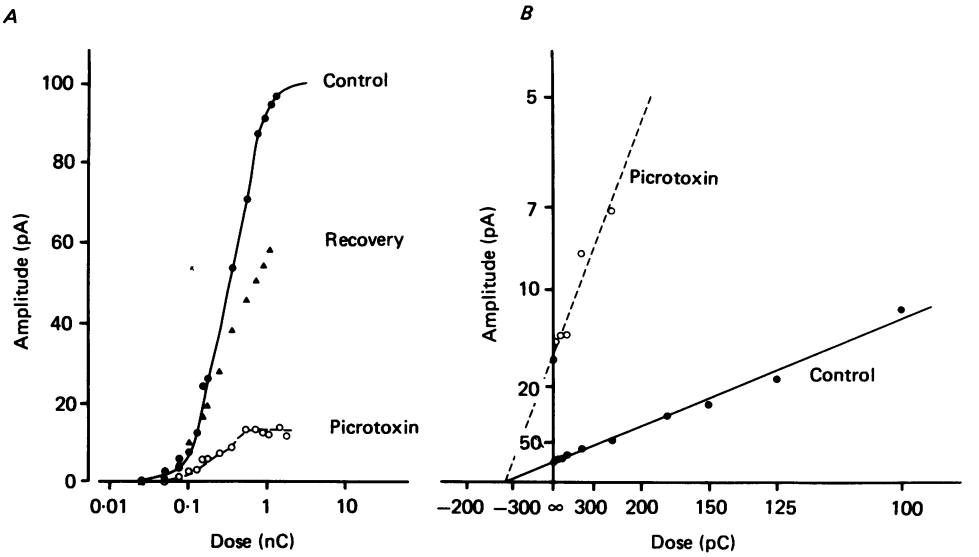

Fig. 10. Effect of picrotoxin on the dose–response relationship of GABA in a cone containing red oil droplet. The same cell as in Fig. 9. *A*, peak amplitude of GABA-induced current plotted on a semilogarithmic coordinate. Control (●), in the presence of 10 μM-picrotoxin (○), after wash-out (▲). *B*, reciprocal of peak amplitude is plotted against reciprocal of the square of dose (by assuming cooperativity of GABA to be 2). Lines are fitted by the least squares methd. K_D for GABA is 275 pC (no picrotoxin) and 266 pC in 10 μM-picrotoxin. I_{max} is 100 pA (no picrotoxin) and 15 pA (in 10 μM-picrotoxin).

One can estimate R_{max} and the amount of dose (K_D) necessary to evoke the half saturating response by plotting the reciprocal of response amplitudes against the reciprocal of the square of dose (square of dose was used on the ordinate, because two GABA molecules are likely to bind to a single GABA receptor). As expected, addition of bicuculline increased K_D, but did not affect R_{max} (Fig. 9*B*), suggesting that bicuculline blocked GABA responses in a competitive manner as demonstrated in earlier binding studies (Zukin, Young & Snyder, 1974).

On the other hand, after the application of 10 μM-picrotoxin, the maximum response decreased in amplitude without a change in the threshold dose or in the saturating dose (Fig. 10*A*). These changes were also represented in the double reciprocal plot as a smaller value of R_{max} and an unaffected K_D (Fig. 10*B*). These results indicate that bicuculline blocked GABA responses in a competitive manner, while picrotoxin blocked GABA responses in a noncompetitive manner, as has been

demonstrated in the crayfish neuromuscular junction (Takeuchi & Takeuchi, 1969).

Effects of GABA agonists. In isolated turtle cones, muscimol was as effective as GABA (least effective dose, *ca.* 100 nM), while baclofen evoked no responses even when a 100 μM dose was applied at various holding potentials ($-106 \sim +34$ mV). These observations, together with the sensitivity to bicuculline and activation of Cl^- conductance (g_{Cl}), suggest that turtle cones have $GABA_A$ receptors.

DISCUSSION

Tachibana & Kaneko (1984) demonstrated that turtle cones are sensitive to GABA and that the sensitivity varies among subtypes. They found that both red-sensitive (except for the accessory member of double cones) and green-sensitive cones have high sensitivity to GABA, while blue-sensitive cones and rods have very low sensitivity. Since monophasic horizontal cells synthesize (Lam, 1972; Lam *et al.* 1979), accumulate (Lam & Steinman, 1971; Marc *et al.* 1978; Schwartz, 1982) and release GABA (Schwartz, 1982; Miller & Schwartz, 1983), it is hypothesized that this amino acid is the transmitter substance of the synapse from monophasic horizontal cell to red-sensitive and green-sensitive cones. The present study, as well as our previous report (Tachibana & Kaneko, 1984), presents another piece of supporting evidence for the GABA hypothesis. Effective concentration of GABA was as low as 100 nM, and the GABA sensitivity was clearly localized at the cone pedicle on which horizontal cell processes terminate. GABA induced an increase in g_{Cl}.

In isolated cones recorded with patch pipettes filled with a solution of $[Cl^-]_p$ 24 mM, a concentration close to their $[Cl^-]_i$, a saturating dose of GABA induced a g_{Cl} increase by about 1·1 nS on average. Desensitization may reduce the amount of GABA-induced g_{Cl} to about $\frac{1}{3}$ of maximum, i.e. 0·4 nS, but this value is still $\frac{1}{6}$ to $\frac{1}{3}$ of the light-sensitive conductance at the cone outer segment (2·5 nS in tiger salamander cone, Attwell, Werblin & Wilson, 1982; 1·3 nS in turtle cones calculated from the data of Schnapf & McBurney, 1980, by assuming the membrane potential to be -30 mV). Therefore, one of the effects of GABA may be to keep the membrane potential near E_{Cl} by shunting other currents generated in the cell. This effect is enhanced if $[Cl^-]_i$ is higher, since larger g_{Cl} values were obtained in cells recorded with higher $[Cl^-]_p$.

In the present experiment, $[Cl^-]_i$ of isolated cones was estimated to be about 20 mM. However, $[Cl^-]_i$ of *in situ* cones could be higher as reported by electron probe analysis (Somlyo & Walz, 1985). If Cl^- distributes passively across the cell membrane, $[Cl^-]_i$ might increase because the dark membrane potential is at a more positive value than the E_{Cl} we estimated due perhaps to a steady inward current through the outer segment. The light-sensitive current was lacking in our preparations in which the outer segments were lost during dissociation. In *in situ* cones of *Pseudemys*, the dark membrane potential is reported to be between -30 and -40 mV (see e.g. Baylor & Hodgkin, 1973). The polarity of GABA-induced response depends both on the membrane potential and E_{Cl}. Although E_{Cl} (or $[Cl^-]_i$) has not yet been estimated in *in situ* cones, the observation of Murakami *et al.* (1982*a*) is a good indication that GABA produces hyperpolarization in *in situ* cones. They recorded 5 mV hyperpolarization of the dark membrane potential (-30 mV) in a green-sensitive cone of the carp retina in response to bath-applied 5 mM-GABA.

It is tempting to speculate on the functional aspects of the GABAergic negative feed-back pathway from monophasic horizontal cells to red-sensitive and green-sensitive cones based on the diagram proposed by Fuortes & Simon (1974). In the dark, cones release their transmitter tonically (Trifonov, 1968). There are a number of reports that the transmitter substance from cone photoreceptors depolarizes horizontal cells (Dowling & Ripps, 1973; Cervetto & Piccolino, 1974; Kaneko & Shimazaki, 1975). The depolarization then triggers release of GABA from monophasic horizontal cells (Schwartz, 1982; Miller & Schwartz, 1983), which, in turn, causes membrane hyperpolarization in both red-sensitive and green-sensitive cones. Illumination hyperpolarizes photoreceptors, and a sequence of events is expected to occur all with the opposite polarity during light flashes.

It seems possible that the negative feed-back interaction plays two important roles in the function of the retina; colour opponent responses of biphasic horizontal cells, and centre-surround antagonism. If the aforementioned sequence of events in the neural chain starts from the red-sensitive cone, and is relayed by the monophasic horizontal cell to green-sensitive cones, then biphasic horizontal cells, at the end of this cascade chain, will respond with depolarization to red light (cf. Fuortes & Simon, 1974). In fact, application of GABA agonists or antagonists to the fish retina affected chromatic responses of biphasic and triphasic horizontal cells (Murakami *et al.* 1982*b*; Toyoda & Fujimoto, 1983). They demonstrated that the red-depolarizing component of biphasic horizontal cells disappeared with application of an excess GABA (perhaps due to saturation) or with application of picrotoxin (due to antagonism).

If and when the negative feed-back operates between red-sensitive cones and monophasic horizontal cells bidirectionally, such interaction would result in surround antagonism in red-sensitive cones, since the spatial summation in monophasic horizontal cells is much larger than in red-sensitive cones. The negative feed-back circuit may also contribute in controlling the gain of the red-sensitive cones (and horizontal cells) by compressing the amplitude of their light-evoked voltage responses.

Depolarization of cones in response to surround illumination depended on the membrane potential of cones as reported by O'Bryan (1973) and by Piccolino & Gerschenfeld (1980). Hyperpolarization of cones by extrinsic current suppressed both the feed-back 'spikes' (Piccolino & Gerschenfeld, 1980) and graded depolarization in response to surround illumination. The observations may be interpreted, at least in part, by a reduction of the driving force for Cl^- based on the present observations. Similar reduction in amplitude of the surround depolarization, or even a polarity reversal, is expected to occur when cones are hyperpolarized by a strong incident light. When inverted, the feed-back effect may be difficult to isolate from the light-evoked hyperpolarization.

Morphological studies have shown that biphasic, and triphasic horizontal cells also have subtype-specific connexions (Leeper, 1978); biphasic cells connect with green-sensitive and blue-sensitive cones, and triphasic cells with blue-sensitive cones. These types of horizontal cells do not seem to use GABA as the transmitter, since they neither accumulate GABA nor have machinery to synthesize GABA (Lam, 1972; Lam & Steinman, 1971; Marc *et al.* 1978; Lam *et al.* 1979). The low GABA sensitivity in blue-sensitive cones (Tachibana & Kaneko, 1984) agrees with those reports, but the

question of how these types of horizontal cells communicate with photoreceptors still remains open.

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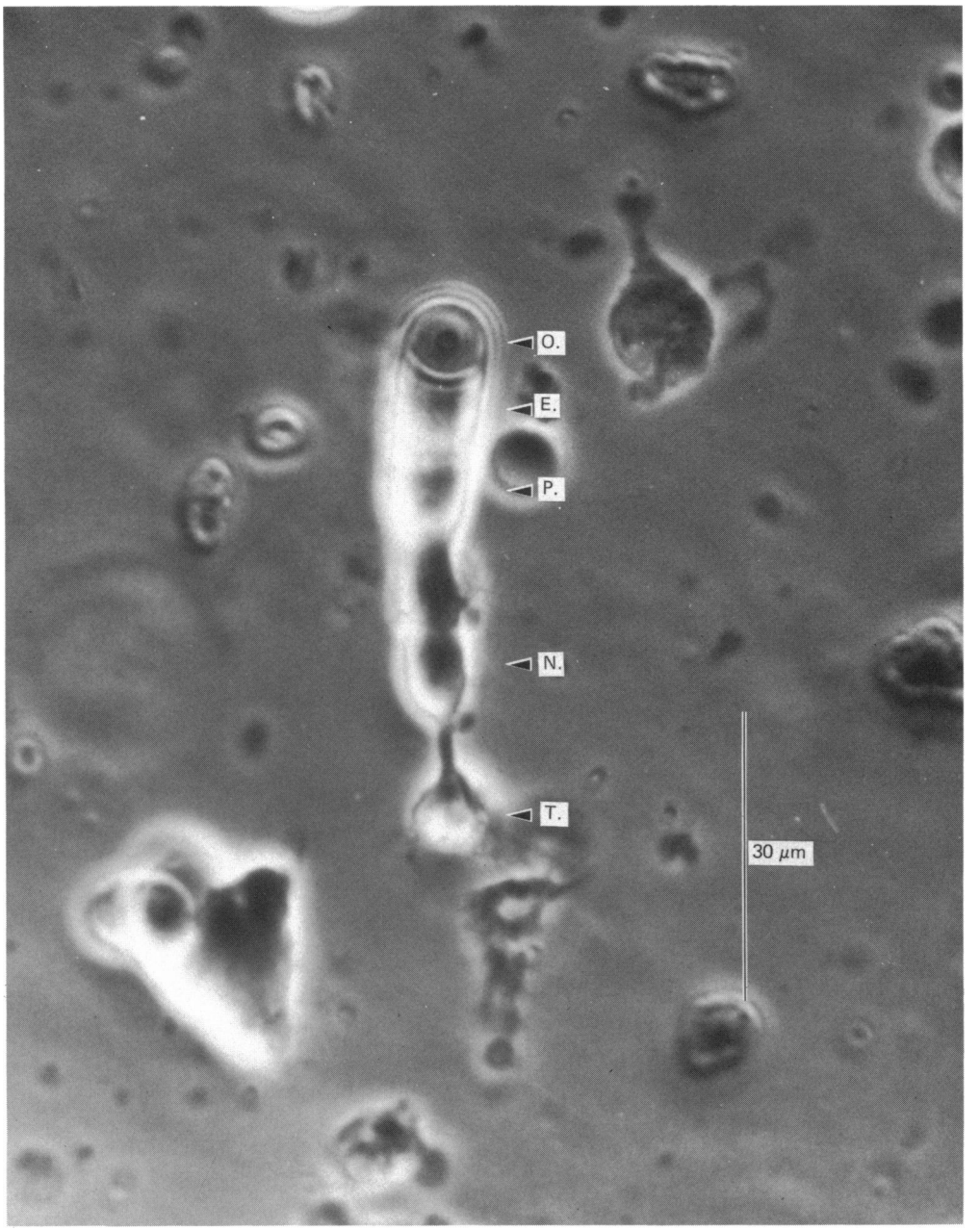
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ZUKIN, S. R., YOUNG, A. B. & SNYDER, S. H. (1974). Gamma-aminobutyric acid binding to receptor sites in the rat central nervous system. *Proceedings of the National Academy of Sciences of the U.S.A.* **71**, 4802–4807.

EXPLANATION OF PLATE

Photomicrograph of a solitary cone containing red oil droplet (red-sensitive cone). O., oil droplet; e., ellipsoid; p., paraboroid; n., nuclear region; t., axon terminal (or cone pedicle). Calibration bar, 30 μm.