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γ-Aminobutyric acid exerts a local inhibitory action on the axon terminal of bipolar cells: Evidence for negative feedback from amacrine cells

(vision/retina/chemical transmitter/synapse/γ-aminobutyric acid type A receptor)

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ABSTRACT **It is well-established morphologically that bipolar cells, the second-order neurons in the vertebrate retina, make reciprocal synapses with amacrine cells in the inner plexiform layer. However, neither the property nor the physiological function of the feedback synapse is understood. Autoradiographic and immunohistochemical studies suggest the presence of γ-aminobutyric acid (GABA)-ergic amacrine cells, and therefore the bipolar cells are thought to receive GABAergic inputs from amacrine cells. This possibility was investigated in the present study, in which we used solitary bipolar cells dissociated from the goldfish retina enzymatically. Dissociated solitary bipolar cells showed a large variety in morphology. In the present study, we selected the bipolar cells with a huge bulbous axon terminal. Bipolar cells of this subtype were identical in morphology to the on-center cells with rod-dominant inputs as revealed in earlier studies by intracellular staining. Membrane currents were measured under voltage clamp with a patch pipette in the whole cell configuration. In some experiments, GABA-sensitive membrane was excised as an outside-out patch from the axon terminal bulb of solitary bipolar cells. All cells of this type responded to GABA. The highest sensitivity was located at the axon terminal. The minimal effective dose was on the order of 10^{-7} M. GABA increased the chloride conductance and evoked a membrane hyperpolarization. Partial desensitization was observed during the application of GABA. The bipolar cells had GABA type A receptors. These results are consistent with the idea that the rod-dominant on-center bipolar cells receive negative feedback inputs from GABAergic amacrine cells.**

Bipolar cells in the vertebrate retina are the second-order neurons that process the signal received from photoreceptors. Bipolar cells have a concentrically arranged receptive field with center-surround antagonism (1, 2). One type of cells (on-center cells) responds with graded and sustained membrane depolarization to illumination of the receptive field center, while the other type of cells (off-center cells) responds with hyperpolarization. Bipolar cells make ribbon synapses with amacrine and ganglion cells, at which bipolar cells send out signals to both neurons. Near the ribbon synapse, numerous terminals are seen making conventional synapses on the bipolar cell terminal (3, 4). These terminals are known to originate from amacrine cells and are thought to mediate negative feedback to bipolar cells. Despite the morphological evidence, the physiological function of the feedback synapse is not understood.

Type Ab amacrine cells, which respond to light with sustained depolarization, are supposed to be γ-aminobutyric acid (GABA)-ergic, since they contain L-glutamic acid decarboxylase, a synthesizing enzyme of GABA (5), and since they accumulate and release GABA (6–8). These types of amacrine cells are assumed to make feedback synapse on the axon terminal of on-center bipolar cells in the sublamina b of the inner plexiform layer (9). The aim of the present study is to investigate the possibility that GABA is the transmitter substance that mediates the feedback signal from type Ab amacrine cells to on-center bipolar cells. To avoid the confusion arising from the complex retinal network, we used solitary bipolar cells dissociated from the goldfish retina. One type of on-center bipolar cells could be unequivocally identified by their characteristic morphology (10, 11). Using this type of bipolar cells, we studied the sensitivity to extrinsically applied GABA and the properties of GABA-induced responses.

MATERIALS AND METHODS

Preparations. Solitary bipolar cells were obtained from the retina of adult goldfish, *Carassius auratus* (body length, ≈15 cm) by the procedure described in detail elsewhere (12). In short, retinas detached from the pigment epithelium were incubated at 28°C for ≈40 min in a solution containing papain (3–15 units/ml; Cooper). They were rinsed with papain-free solution and triturated mechanically by pipetting with a large-tipped Pasteur pipette. Dissociated cells were plated in a culture dish. The bottom of the dish was drilled (diameter, ≈12 mm), where a concanavalin A-coated cover glass was attached. Experiments were performed on freshly isolated cells within 10 hr after dissociation.

Dissociation yielded a mixture of retinal neurons but bipolar cells were easily identified from their typical morphology. Solitary bipolar cells showed a large variety in morphology. Among those, we often encountered cells of a particular subtype as shown in Fig. 1. Since cells of this subtype had a huge axon terminal while others did not, their identification was unequivocal. Saito and colleagues (10, 11) have made a systematic study to correlate morphology with physiology of cyprinid fish bipolar cells by intracellular staining and have shown that only rod-dominant on-center cells have a huge bulb-like axon terminal. In reference to their work, solitary bipolar cells with a huge axon terminal were identified to be the rod-dominant on-center cells. We confined our present study to cells of this subtype to avoid variation of data due to the heterogeneity of cell types.

Recording Procedures. A culture dish was mounted on the stage of an inverted microscope with phase-contrast optics (Nikon TMD, Japan). Solitary cells were continuously superfused with a solution containing 120 mM NaCl, 2.6 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, and bovine serum albumin at 0.1 mg/ml (pH 7.4, 15°C). Cells were photographed prior to recording. Membrane currents were measured under voltage clamp with a

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Abbreviation: GABA, γ-aminobutyric acid.

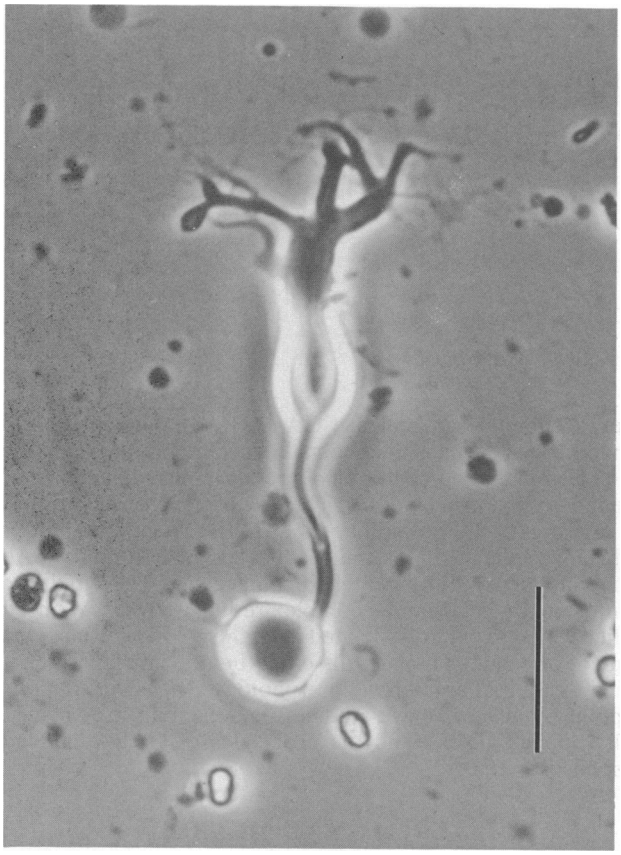

FIG. 1. Phase-contrast micrograph of a solitary bipolar cell from the goldfish retina. The cell is identified as the rod-dominant on-center type by its characteristic morphology: thick dendrites, large cell body, stout axon, and large axon terminal (10). (Bar = 20 μm.)

patch pipette in the whole cell configuration (13). In some experiments, GABA-sensitive membrane was excised as an outside-out patch from the axon terminal bulb of solitary bipolar cells. The control pipette solution contained 120 mM KCl, 5 mM EGTA, and 10 mM Hepes and the pH was adjusted to 7.2 with KOH (final concentration, 13 mM). In some cases, KCl was replaced with equimolar CsCl to block K currents (the Cs pipette solution). A patch electrode, resistance of which was ≈20 MΩ in the superfusate, was connected to a low noise current-voltage converter (List Electronics, L/M EPC 7 or Nihon Koden CEZ-2100). The data were displayed on an oscilloscope and on a pen recorder and were appropriately filtered and sampled by an A/D converter connected to a computer (VAX11/750). The liquid junction potentials at the patch electrode and at the indifferent electrode were measured and corrected for as described elsewhere (14).

Drug Application. GABA was applied ionophoretically from a fine glass pipette (conventional intracellular recording pipette) filled with 1 M GABA (pH 4.0 adjusted with HCl). The GABA pipette was placed against the cell surface and a small amount of GABA was ejected locally by passing a brief current pulse to the GABA pipette. A small amount of direct current of the opposite polarity (brake current) minimized the diffusion of GABA from the pipette tip. In some cases, a known concentration of GABA was dissolved in the superfusate and was pressure-applied to the recorded cell so that the test solution could soak the entire cell surface (15). Bath application was also used when necessary. Muscimol (Sigma) or baclofen (a gift from CIBA-Geigy) was dissolved in the superfusate and was pressure-applied. Bicuculline methochloride or picrotoxin (Sigma) was bath-applied.

RESULTS

Distribution of GABA Sensitivity. All solitary bipolar cells with a huge terminal bulb (total, 139 cells) responded to extrinsically applied GABA. As shown in Fig. 2*A*, a small amount of GABA (intensity of ionophoretic current, 30 nA; duration, 3 ms) evoked a large inward current in a cell voltage-clamped at −66 mV.

Local application of GABA revealed that the sensitivity to GABA was the highest at the terminal bulb region (Fig. 2*A*). In this example, GABA did not evoke discernible responses when applied to the dendritic or somatic region. However, an increase in dose revealed that the distal parts of the cell were also sensitive to GABA. For example, GABA at 10 times as large dose as that of Fig. 2*A* given to the dendritic tip evoked 12 pA inward current, which was ≈3% of the response evoked when the identical dose of GABA was applied to the terminal bulb. When the same amount of GABA was applied to the cell body, it evoked a response whose amplitude was 20% of that evoked at the terminal bulb. These observations suggest (*i*) that high GABA sensitivity is confined to the terminal bulb, and (*ii*) that the distal parts of the cell are also sensitive to GABA to some extent.

Dose–Response Relationship. The amplitude of the GABA-induced responses was dose dependent. Fig. 2*B* illustrates the dose–response relationship measured at the terminal bulb. As the dose of GABA was increased, the response amplitude became larger and finally reached a saturation. The average saturating response of 139 cells was 568 ± 311 pA (mean ± SD; holding potential, −66 mV). The dose–response relationship was sigmoidal over a range of 0.5–1.0 log unit of dose with a Hill coefficient of ≈3.5, indicating that GABA has a high cooperativity. Measurement of the dose–response relationship at the cell body revealed that the minimal effective dose of GABA to the cell body was nearly identical to that to the terminal bulb.

To estimate the minimal effective concentration of GABA, GABA was dissolved in the superfusate at various concen-

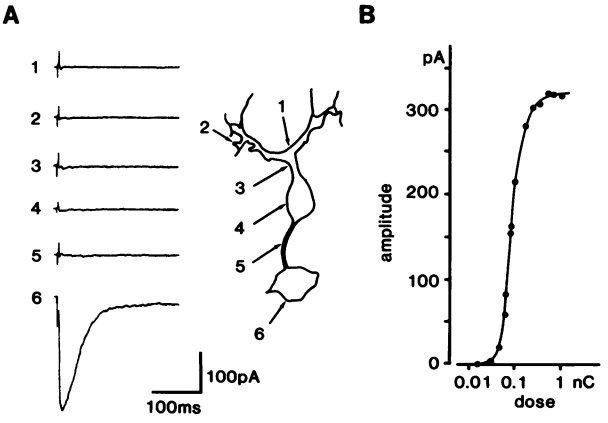

FIG. 2. GABA-induced responses in a solitary bipolar cell. (*A*) Mapping of GABA sensitivity. Identical doses of GABA were applied by ionophoresis (30 nA × 3 ms; brake current, −5 nA) from a fine-tip pipette positioned at various parts of the cell, indicated by arrows. Holding potential, −66 mV. The label number of each current trace corresponds to the position of the pipette for ionophoresis. The biphasic transient deflection of current traces is the artifact due to the ionophoretic current pulse. (*B*) Dose–response relationship obtained from the cell shown in *A*. GABA was applied ionophoretically to the axon terminal of the cell voltage-clamped at −66 mV. The dose is expressed by the product of the intensity and duration of the ionophoretic pulses. The peak amplitude is plotted against the dose on a logarithmic scale.

trations and pressure-applied from a pipette (tip diameter, ≈20 μm) to soak the recorded cell entirely. GABA at 100 μM evoked a saturating response, while 1 μM GABA evoked a small response (10–20% of the saturating response). By an extrapolation of the above observations, the minimal effective concentration was estimated to be on the order of 10^{-7} M.

Ionic Mechanism of the GABA Response. The species of ions that carried GABA-induced current was identified from the reversal potential of this current. In a series of experiments, we recorded from an outside-out membrane patch excised from the terminal bulb (Fig. 3*A*) to reduce current fluctuations (16) due to voltage-dependent and Ca-dependent currents. Ionophoretic application of GABA induced a large inward current (>200 pA) in the membrane patch voltage-clamped at −46 mV. Fluctuation in current increased during the GABA-induced response, which was seen at higher gain (see Fig. 6), but no single-channel event was resolved. A large GABA-induced current indicates that the patch membrane contained a high density of GABA receptors. As the membrane was depolarized, the GABA-induced inward current decreased in amplitude, and at ≈0 mV the response polarity was reversed to the outward direction. The relationship between the peak amplitude of responses and the membrane potential was almost linear and the reversal potential was −4 mV (Fig. 3*B*, solid circles). Thus, we inferred that GABA increased the membrane conductance.

Similar measurements were made by exposing the outside of this membrane to solutions that contained various concentration of Cl^- ($[Cl^-]_o$) (Fig. 3*B*). The reversal potential was shifted to a more positive value at lower $[Cl^-]_o$. The accumulated data on 15 patch membranes were plotted against $[Cl^-]_o$ (Fig. 4). The data points were fitted very well to the equilibrium potential for Cl^-. Changes in $[Na^+]_o$ or $[K^+]_o$ caused no effect on the reversal potential of the GABA-evoked responses. These observations suggest that Cl^- carried the GABA-induced current. We made similar experiments on isolated bipolar cells in the whole cell clamp

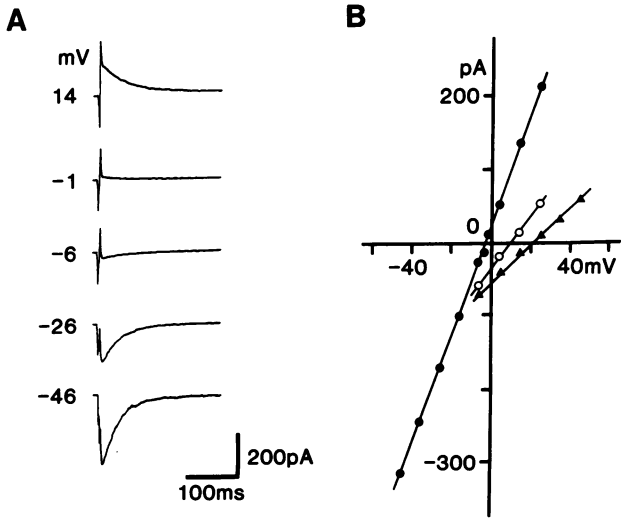

FIG. 3. Reversal potential of GABA-induced responses. An outside-out patch membrane was excised from the axon terminal and voltage-clamped. GABA was applied ionophoretically (50 nA × 5 ms; brake current, −5 nA). (*A*) Reversal of GABA-induced currents. Holding potentials are indicated to the left of each current trace. The transient biphasic deflection of current traces is the artifact due to the ionophoretic current pulse. $[Cl^-]_o$ = 133 mM, $[Cl^-]_{pipette}$ = 120 mM. (*B*) Relationship between GABA-induced current and membrane potential. Outside patch membrane was superfused with a solution containing 133 mM Cl^- (●), 67 mM Cl^- (○), or 40 mM Cl^- (▲). Chloride ions were replaced with methanesulfonate ions.

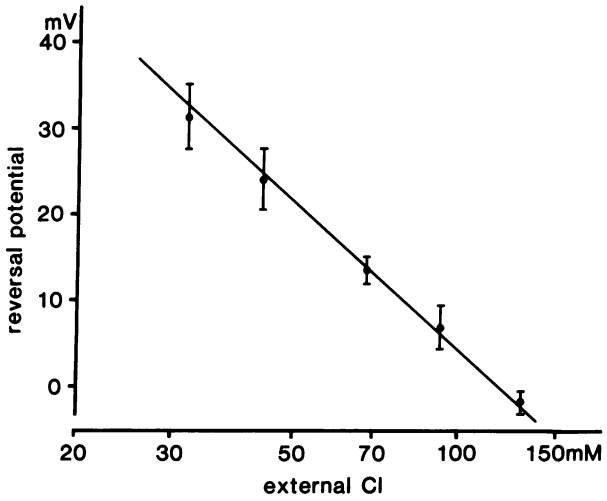

FIG. 4. Relationship between reversal potential and $[Cl^-]_o$. Data were obtained from 15 outside-out patch membranes excised from the terminal region. Means ● ± SD (vertical bars) are shown. Line represents equilibrium potential for Cl^- calculated by the Nernst equation ($[Cl^-]_{pipette}$ = 120 mM).

configuration by using patch pipettes filled with the Cs pipette solution (to suppress the K currents). Results obtained in this series of experiments were substantially the same as in the experiments on excised patches.

Polarity of GABA-Induced Response. To relate our data to the physiological function of the GABAergic feedback synapse, it is essential to know whether GABA evokes membrane hyperpolarization or depolarization in bipolar cells. The aforementioned observation that the reversal potentials of GABA-induced currents were identical both in the excised patch and in the whole cell suggests that the cytoplasm of the recorded cell was dialyzed by the pipette solution of the patch electrode nearly completely. However, it also seems reasonable to assume that dialysis requires a certain length of time. Therefore, immediately after the rupture of the patch membrane the intracellular ionic concentration should remain unchanged from the value in unruptured cells. On this assumption, we measured the voltage responses induced by GABA during this period. An example is shown in Fig. 5. The resting potential of this cell was −36 mV. An application of GABA 3 s after the rupture evoked a hyperpolarizing response reaching approximately −60 mV. Successive application (interval, 10 s) of GABA revealed that the hyperpolarizing response decreased in amplitude with time and finally the response polarity changed to depolarization. Successive changes in response amplitude and the polarity reversal are

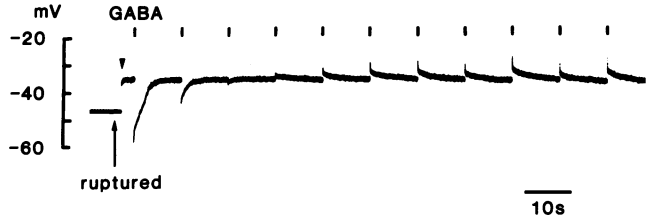

FIG. 5. Voltage responses to GABA immediately after rupture of patch membrane. First, GΩ seal was established with a patch pipette containing 120 mM Cl^-. As soon as the patch membrane was ruptured (arrow), the recording system was switched from the voltage-clamp mode to the current-clamp mode (arrowhead) to measure the membrane potential. GABA was applied repetitively once every 10 s to the axon terminal by ionophoresis (30 nA × 10 ms; brake current, −5 nA) as indicated by vertical bars. The resting membrane potential was −36 mV.

well ascribed to the diffusion of Cl^- into the cell from the recording pipette. The result indicates that GABA evokes hyperpolarizing responses in unruptured bipolar cells. Therefore, it seems very likely that GABA exerts an inhibitory effect on *in situ* bipolar cells.

Desensitization. Desensitization of postsynaptic receptors gives a strong influence to the temporal nature of synaptic transmission. In our case, for example, even if the input from amacrine cells is tonic, it would produce only a phasic inhibition if the GABA receptors of bipolar cells desensitize quickly. To clarify the temporal changes in sensitivity of GABA receptors, GABA was applied by a long ionophoretic pulse (duration, 15 s) to an excised outside-out patch (Fig. 6). A small dose of GABA (intensity, ≤2 nA) evoked a response that was sustained during the application. Increase in dose (3 nA) evoked a larger response at the onset of application, but within a few seconds the response amplitude decayed to a smaller level, which was maintained thereafter. The transient component became more prominent when a saturating dose (10 nA) of GABA was applied. The ratio of the steady-state amplitude to the peak amplitude of this response was 0.2–0.3. We inferred that the reduction of response amplitude with time is due to desensitization and not to a reduction of the driving force, because it was found that the reversal potential of the steady state was almost identical to the reversal potential of the response peak. The results suggest that the GABA receptors of bipolar cells are able to respond to GABAergic inputs even after a partial desensitization.

Effects of Agonists and Antagonists. GABA receptors of isolated bipolar cells were identified as type A ($GABA_A$). Muscimol was equally effective as GABA, and the reversal potential of the muscimol-induced response (-5.4 ± 3.7 mV; $n = 15$) was identical to that of the GABA-induced response. Baclofen, a $GABA_B$ agonist, was ineffective (examined in seven cells). GABA was antagonized by either bicuculline or picrotoxin, but the underlying mechanism was different; bicuculline blocked competitively, while picrotoxin blocked noncompetitively.

It was reported that the GABA-induced response of turtle photoreceptors was blocked by a low concentration (1–10 μM) of Co^{2+} noncompetitively (17). However, GABA-evoked responses of goldfish bipolar cells were not affected by Co^{2+} even when the concentration was increased to 1 mM.

DISCUSSION

The present study demonstrated that GABA hyperpolarized goldfish bipolar cells with a huge exon terminal by increasing the Cl conductance. Similar GABA-induced hyperpolarization has been reported in skate isolated bipolar cells (18). Our present study also showed that $GABA_A$ receptors were densely accumulated at the axon terminal. The bipolar cells we examined were identified as rod-dominant on-center type by the characteristic morphology (10, 11). The present results provide strong physiological evidence that rod-dominant on-center bipolar cells receive negative feedback inputs from GABAergic type Ab amacrine cells in the sublamina b of the inner plexiform layer. Our results are consistent also with the previous report (19) that *in situ* on-center bipolar cells were hyperpolarized by a local application of GABA close to the terminal region.

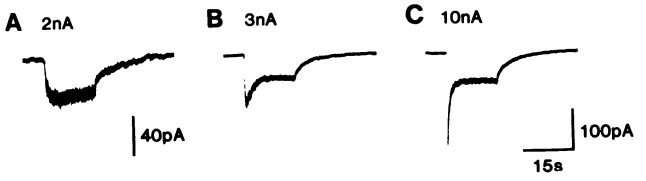

FIG. 6. Desensitization of GABA-induced responses. An outside-out patch membrane was excised from the terminal region and voltage-clamped at −66 mV. GABA was applied for 15 s by ionophoresis (brake current, −5 nA) with the amount of current indicated above each response. Current calibration scale of 100 pA should be applied to *B* and *C*.

It is tempting to speculate how the GABAergic feedback pathway may contribute to the light-evoked response of bipolar cells. Depolarization of on-center bipolar cells by the illumination of receptive field center would be transmitted to Ab (GABAergic) amacrine cells via sign-conserving synapses (20). Depolarization of Ab amacrine cells, in turn, causes a release of GABA (21–23), and the released GABA produces a hyperpolarizing effect on the bipolar cells. GABA-induced hyperpolarization would then be summed with the depolarizing photoresponse of bipolar cells. Thus, the neural circuit as a whole includes a negative feedback, and may contribute to expand the operating range of bipolar cell synapses. This scheme suggests to us that the activity of amacrine cells would cause a detectable inhibitory effect in bipolar cells, but no convincing demonstration of such effects has been made so far. The lack of clear inhibition could be attributed to several reasons. First, it is technically difficult to demonstrate the amacrine-mediated inhibition in bipolar cells by stimulating amacrine cells selectively by light without activating other inhibitory elements, such as horizontal cells. It is reported that both amacrine cells and horizontal cells in the fish retina have receptive fields of similar sizes (2). Second, the driving force (difference between the resting potential and E_{Cl}) of inhibitory postsynaptic potential in *in situ* bipolar cells may be too small to detect the effect of amacrine cells. Third, potentials evoked at the axon terminal could be diminished during electrotonic propagation to the recording site, which is most likely the cell body (24). The input resistance of solitary bipolar cells was found to be high (16), but in the retina it may be much lower due to tonic synaptic inputs or due to electrical coupling between neighboring bipolar cells (25).

When GABA was applied to the dendritic or somatic region, a small response was evoked. The maximal amplitude was ≈10% of that evoked at the axon terminal. Since a subclass of horizontal cells is shown to be GABAergic (5, 6, 26–29), one might argue that bipolar cells could receive a direct input from GABAergic horizontal cells in the outer plexiform layer. However, this possibility seems unlikely because the sensitivity to GABA was very low. In our previous study (30), we found that cone photoreceptors of the turtle retina are highly sensitive to GABA and that the GABA sensitivity is confined to the cone pedicle. The maximum amplitude of the GABA-induced responses and the minimal effective concentration was comparable to that found in the terminal bulb of bipolar cells. It seems therefore more likely that signals from horizontal cells converge to bipolar cells through the horizontal → cone feedback synapse. In fact, GABA ejected ionophoretically at the outer plexiform layer produced a depolarization in on-center bipolar cells in the control retina (hyperpolarization in cones is transmitted to the on-center bipolar cell via sign-inverting synapse), but the effect of GABA was abolished after the chemical synapses were blocked by an application of Co^{2+} (19).

Recent immunohistochemical study has shown that the reactivity against the glutamic acid decarboxylase, a GABA-synthesizing enzyme, was found diffusely in the inner plexiform layer of the goldfish retina (7, 31). Since the processes of off-type cells are confined to sublamina a, while those of on-type cells are confined to sublamina b (9), the report of Yazulla *et al.* (31) may suggest that off-center bipolar cells also receive GABAergic inputs from amacrine cells. GABA sensitivity in off-center cells could also be studied if some criteria for identifying this subtype of bipolar cells becomes available.

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