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Pharmacology of Novel GABA Receptors Found on Rod Horizontal Cells of the White Perch Retina

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A novel type of GABA receptor is present on rod-driven (H4) horizontal cells of the white perch retina (Qian and Dowling, 1993a). These receptors have been tentatively termed $GABA_C$ receptors. In this study, the pharmacological properties of these receptors were further investigated by applying several conformationally restricted $GABA_A$ receptor agonists, $GABA_A$ antagonists, and a $GABA_B$ agonist to the H4 horizontal cells. GABA analogs locked in a partially folded conformation had a variety of effects. Isonipecotic acid had no effect on these receptors, whereas isoguvacine activated them but with low potency (EC_{50} = 137 μM). THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) acted as a competitive antagonist on these receptors with an inhibition constant of 82.5 μM. P4S (piperidine-4-sulfonic acid) activated the receptors at high concentrations (>1 mM), but at lower concentrations it was a competitive antagonist with an inhibition constant of 80.9 μM. I4AA (imidazole-4-acetic acid), a GABA analog with an extended conformation, potently inhibited the GABA responses on H4 horizontal cells with an inhibition constant of 1.67 μM. Muscimol, which can assume both partially folded and extended conformations, acted as a mixed agonist–antagonist. The GABA responses on H4 horizontal cells were resistant to several competitive $GABA_A$ receptor antagonists including bicuculline, hydrastine, and SR-95531, but they were very sensitive to picrotoxin (IC_{50} = 237 nM). The inhibition by picrotoxin was both competitive and noncompetitive in nature. On the other hand, TBPS (*tert*-butylbicyclophosphorothionate), another $GABA_A$ receptor channel blocker, had minimal effects on these receptors. The specific $GABA_B$ agonist 3-APA (3-aminopropyl phosphonic acid) acted as a competitive antagonist on H4 horizontal cells with an inhibition constant of 43 μM. The GABA responses on the horizontal cells were also resistant to strychnine, a glycine receptor antagonist. The results provide further evidence that the GABA receptors on H4 horizontal cells in perch are pharmacologically distinct from known GABA receptors, supporting the designation of $GABA_C$ receptors for them.

[Key words: $GABA_C$ receptor, retina, horizontal cells, pharmacology, GABA]

GABA is the main inhibitory neurotransmitter in the vertebrate CNS. Two GABA receptor types, $GABA_A$ and $GABA_B$, have been well characterized. $GABA_A$ receptors are ligand-gated chloride channels usually linked to fast synaptic inhibition. Such receptors are bicuculline sensitive and often can be allosterically modulated by both barbiturates and benzodiazepines (Olsen, 1982; Bormann, 1988; Sivilotti and Nistri, 1991). $GABA_B$ receptors are coupled to potassium and/or calcium channels through G-proteins, and their responses ordinarily have a slow time course (Gage, 1992). $GABA_B$ receptors can be selectively activated by baclofen and antagonized by phaclofen and 2-hydroxysaclofen (Hill and Bowery, 1981; Bormann, 1988; Kerr et al., 1988; Sivilotti and Nistri, 1991).

In addition to these two well-known types of GABA receptors, the literature suggests another GABA receptor type is present in the vertebrate CNS. Johnston and colleagues (Johnston et al., 1975; Drew et al., 1984) reported a bicuculline- and baclofen-insensitive GABA binding site on rat cerebellar membranes that was antagonized by CACA (*cis*-aminocrotonic acid), a conformationally restricted GABA analog. Such novel GABA binding sites were also reported in the catfish brain (Myers and Tunnicliff, 1988). Johnston (1986) proposed that the receptors mediating these novel responses be termed $GABA_C$ receptors. An unusual GABA response was also reported in central visual pathways by Arakawa and Okada (1988) and Sivilotti and Nistri (1989), who showed that such responses are bicuculline- and baclofen-insensitive, do not show desensitization even following sustained exposure to GABA, and are dependent on extracellular chloride (Nistri and Sivilotti, 1989).

More recently, a bicuculline-resistant GABA response was observed by Polenzani et al. (1991) in *Xenopus* oocytes expressing mRNA derived from bovine retinas. Such responses were sustained and had a higher sensitivity to GABA than typical $GABA_A$ receptor responses. This GABA response could not be modulated by barbiturates and benzodiazepines, and could not be activated by baclofen or suppressed by saclofen. At about the same time, Cutting et al. (1991) using primers derived from a highly conserved region of $GABA_A$ and glycine receptor genes, cloned a GABA receptor (ρ1) subunit from a human retinal cDNA library that, when expressed in *Xenopus* oocytes, formed a homooligomeric GABA receptor. Shimada et al. (1992) showed that the responses mediated by these receptors resemble closely the properties of the GABA responses recorded from oocytes expressing retinal mRNA, and they proposed that the receptors made up of ρ1 subunits are the $GABA_C$ receptors.

We have recorded GABA responses from rod-driven (H4) horizontal cells of the white perch retina that are sustained and mediated by the opening of chloride channels (Qian and Dowl-

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ing, 1992, 1993a). These responses are bicuculline insensitive and not modulated by either barbiturates or benzodiazepines. They are also baclofen insensitive and cannot be blocked by either phaclofen or 2-hydroxysaclofen. Thus, these responses resemble the putative $GABA_C$ responses recorded by Polenzani et al. (1991) and by Shimada et al. (1992).

More detailed studies on the receptors in *Xenopus* oocytes made up of the $\rho 1$ subunit has revealed a unique pharmacology (Kusama et al., 1993; Woodward et al., 1993). For example, most $GABA_A$ agonists act as antagonists on *Xenopus* oocytes expressing the $\rho 1$ subunit. The detailed pharmacology of the novel GABA receptors on the H4 horizontal cells of the white perch retina is not known. These cells provide an excellent system in which to study the properties of these novel receptors in neurons, since the GABA responses on H4 horizontal cells seem to be mediated solely by these novel receptors (Qian and Dowling, 1993a). Although similar GABA responses have been observed in other retinal neurons, in all other cases reported so far the responses are mixed with the responses of other GABA receptors and/or GABA transporters (Dong and Werblin 1993; Feigenspan et al., 1993; Lukasiewicz et al., 1993; Qian and Dowling, 1993b). In this study, the pharmacological properties of GABA receptors on H4 horizontal cells of the white perch retina were characterized with agents known to interact with classic $GABA_A$ and $GABA_B$ receptors.

Materials and Methods

Cell isolation. Solitary retinal cells were isolated from the white perch retina using methods described previously (Dowling et al., 1985). Briefly, eyes were enucleated, rinsed with 70% ethanol, and hemisected at the ora serata. The retina was isolated using fine forceps, and incubated for 40 min in an enzymatic solution made up of Leibovitz's L-15 culture media (GIBCO–Bethesda Research Labs Life Technologies Inc., Gaithersbug, MD) containing 25 U/ml papain (Worthington Biochemical Corporation, Freehold, NJ) and a few crystals of *l*-cysteine. The tissue was washed five times with fresh L-15 and triturated through a sterilized pipette. Aliquots of the supernatant containing the isolated cells were placed in 35 mm petri dishes containing 3 ml of L-15 culture medium with no antibiotics added. The dishes were left in the dark at room temperature for up to 1 week.

Electrical recordings. Before each experiment, the culture medium was replaced with Ringer's solution, containing (mM) choline Cl (145), $CaCl_2$ (2.4), $MgCl_2$ (1.5), glucose (10), and HEPES (10). The whole-cell version of the patch-clamp recording technique was used to record membrane current from horizontal cells (Hamill et al., 1981). Pipettes with tip diameters of about 1 μm were pulled on a Narishige two-step puller. Recording pipettes were filled with an intracellular solution containing (mM) CsCl (124), $CaCl_2$ (1), EGTA (11), $MgCl_2$ (2), HEPES (10). A patch-clamp amplifier (Dagan Corporation, Minneapolis, MN), linked to an IBM-PC 486 computer, was used to measure membrane current recorded from the cell. The voltage-clamp procedure was controlled using the software PCLAMP (Axon Instruments Inc., Burlingame, CA). Cells were held at −50 mV. Membrane potentials were corrected for electrode tip potential as in Fenwick et al. (1982). Recordings were stored both on the computer and on video tape using a Vetter PCM recorder. The resistance of a typical patch pipette was about 2 MΩ, and series resistance during whole-cell recording was about 4 MΩ. Consequently, a 100 pA current would produce an error of 0.4 mV in horizontal cell membrane voltage. Recordings were not compensated.

Drug delivery. A local superfusion system was used to deliver drugs onto the cells (Qian et al., 1993). Drugs were added at various concentrations to Ringer's solution and introduced into the culture dish through a pipette (tip diameter ~ 200 μm) via a gravity-fed superfusion system at a rate of about 1 ml/min. The pipette was mounted on a micromanipulator and positioned about 500 μm from the cells. The perfusate was removed from the culture dish by a suction-operated siphon. For experiments with antagonists, drugs were coapplied with GABA. GABA and I4AA were obtained from Sigma Chemical Co. (St. Louis, MO). All other agents were obtained from Research Biochemicals Inc. (Natick, MA).

Data analysis. McPherson (1989) has presented a detailed mathematical analysis of drug–receptor interactions. Briefly, the relation of drug concentration and response of any receptor mediated response can be described by the Hill equation:

$$\frac{R}{R_{max}} = \frac{[C]^n}{[C]^n + EC_{50}^n}, \quad (1)$$

where R is the response of the cell induced by the drug at concentration $[C]$, R_{max} is the maximum response of the cell, n is the Hill coefficient, and EC_{50} is the concentration at which a half-maximum response is induced. On white perch H4 horizontal cells, 100 μM GABA induces maximal responses (see Fig. 1*B*).

The dose–response curve for antagonists can be described by the equation

$$\frac{R}{R_0} = 1 - \frac{[I]^n}{[I]^n + IC_{50}^n}, \quad (2)$$

where R_0 is the GABA response without antagonist, R is the response induced by GABA in the presence of antagonist, $[I]$ is the concentration of the antagonist, n is Hill coefficient, and IC_{50} is the concentration of antagonist at which half of GABA response is inhibited. To elicit sizable responses, 10 μM GABA was used as the test concentration.

For a competitive antagonist, the value of IC_{50} can be used to estimate the inhibition constant (K_i) of the antagonist. However, the relation of K_i and IC_{50} depends on the concentration of agonist used for the test, which can be expressed as follows:

$$K_i = \left(\frac{R_{max} - R_0}{R_{max}}\right)^{1/n} IC_{50}, \quad (3)$$

where R_0 is the test response (same as R_0 in Eq. 2), that is, the response induced by 10 μM GABA. For the GABA receptors on H4 horizontal cells (with 10 μM GABA as test) $K_i = 0.286\ IC_{50}$ (see Fig. 1*B*).

A more precise way to determine the inhibition constant of competitive antagonists is to measure the shift of EC_{50} value of the dose–response curve in the presence of a certain concentration of the antagonist. K_i can be determined as follows:

$$K_i = \frac{[I]}{\left(\frac{EC_{50[I]}}{EC_{50}} - 1\right)}, \quad (4)$$

where $EC_{50[I]}$ is the EC_{50} for dose–response relationship of GABA in the presence of the antagonist at concentration $[I]$.

For noncompetitive antagonists, the dose–response curve can be expressed as

$$\frac{R}{R_{max}} = M^* \frac{[C]^n}{[C]^n + EC_{50}^n}, \quad (5)$$

where M is the maximum response remaining in the presence of the antagonist.

The experimental data were fitted to the theoretical equations by the commercial software program SIGMAPLOT (Jandel Scientific Inc., San Rafael, CA). All data are expressed as means ± SD.

Results

Properties of GABA responses on H4 horizontal cells

Figure 1*A* shows the responses of a white perch H4 horizontal cell to a 30 sec pulse of GABA at several different concentrations. When the cells were held at −50 mV, GABA induced an inward current. We have shown previously that this current is carried by chloride ions (Qian and Dowling, 1993a). The current responses have a slow time course, and they show no sign of desensitization during the prolonged GABA application even at the highest concentrations (30–100 μM). A dose–response curve averaged from 15 cells is shown in Figure 1*B*. The continuous curve is a theoretical fit of the data to Equation 1 with $n = 1.83$ and $EC_{50} = 3.03$ μM. These results are in good agreement with our earlier data (Qian and Dowling, 1993a).

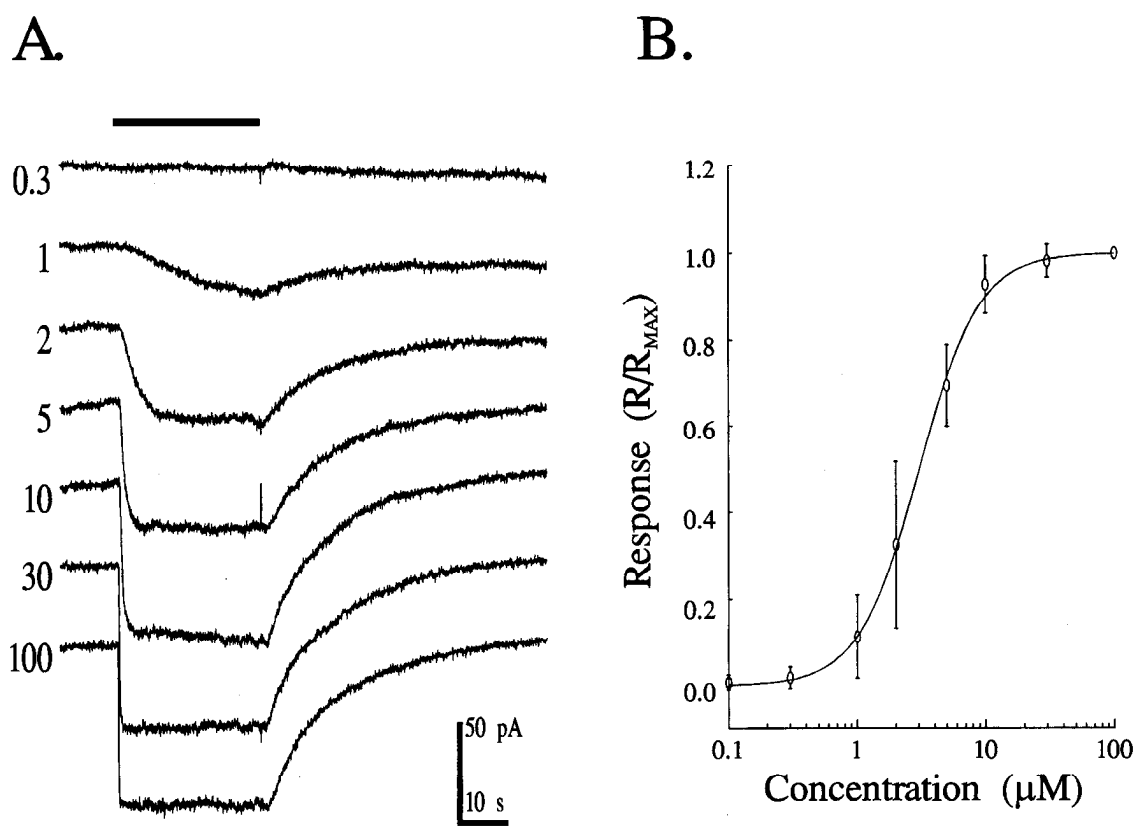

Figure 1. *A*, GABA elicited current responses in an H4 horizontal cell. The concentration of GABA is shown on the *left* of each trace (μM). The duration of GABA application is shown by the *bar* at the *top*. *B*, Dose–response curve for GABA induced currents in H4 horizontal cells. The data are from 15 cells. For each cell, the responses were expressed as a fraction of the maximum response induced by 100 μM GABA, R_{max}. The continuous curve is a mathematical fit of data points to the Hill equation with the parameters of $n = 1.83$, $EC_{50} = 3.03$ μM.

$GABA_A$ receptor agonists

Drug rationale. The GABA molecule exhibits a considerable conformational flexibility because each single bond within the molecule can rotate freely. Different conformations of GABA may interact with different types of receptors (Johnston, 1992). Previous studies have shown that GABA interacts best with $GABA_A$ receptors in a partially folded, planar conformation as shown in Figure 2*A* (Krogsgaard-Larsen et al., 1985; Johnston, 1992). Indeed, compounds with such a restricted conformation are all potent $GABA_A$ receptor agonists, and several such compounds that were used in this study are shown in Figure 2*A*. Isonipecotic acid is an amino group locked GABA analog, whereas isoguvacine and P4S (piperidine-4-sulfonic acid) are derivatives of isonipecotic acid. THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) is an amino group locked muscimol analog.

GABA can also assume a fully extended conformation as shown in Figure 2*B*, and I4AA (imidazole-4-acetic acid) is an analog of such conformation. I4AA is a $GABA_A$ receptor agonist, but it is not as potent as the compounds in a partially folded conformation. Muscimol, on the other hand, can exist in both the fully extended and partially folded conformation since the N–C bond is not locked. These two conformations of muscimol are shown in Figure 2*C*.

Partially folded analogs. Figure 3*A* shows the effects of GABA (10 μM), isoguvacine (100 μM), and isonipecotic acid (100 μM) on an H4 horizontal cell isolated from the white perch retina. Both GABA and isoguvacine induced a slow sustained inward current in the cells, although the response generated by 100 μM isoguvacine was only about one-third the amplitude of the response generated by 10 μM GABA. Interestingly, the responses induced by isoguvacine returned to the baseline much faster than did the responses induced by GABA. This has also been observed in responses induced by CACA and muscimol (Qian and Dowling, 1993a), and may reflect the characteristics of the binding of these agents to these receptors.

Isoguvacine can fully activate the receptor on H4 horizontal cells, as shown by the dose–response curve for isoguvacine in Figure 3*B*. However, isoguvacine ($EC_{50} = 137$ μM) is much less potent on H4 horizontal cell receptors than is GABA. This contrasts with its action on classic $GABA_A$ receptors, which are more sensitive to isoguvacine than to GABA (Krogsgaard-Larsen et al., 1977). Isonipecotic acid has a very similar molecular structure to isoguvacine (Fig. 2*A*) and also strongly activates $GABA_A$ receptors (Bowery et al., 1978; Krogsgard-Larsen et al., 1985). However, isonipecotic acid (100 μM) elicited no responses from H4 horizontal cells (Fig. 3*A*), nor did it have any effects on GABA induced responses (data not shown).

THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) is a $GABA_A$ receptor agonist that is more potent than GABA in activating $GABA_A$ receptors (Krogsgaard-Larsen et al., 1977; Alger and Nicoll, 1982). No responses were elicited in the horizontal cells when THIP was presented alone. When THIP was coapplied with GABA, it reduced the GABA responses as shown in Figure 4*A*. The responses are expressed as a fraction of the response induced by 10 μM GABA in Ringer's, and can be described by Equation 2, with parameters of $n = 2.1$ and IC_{50}

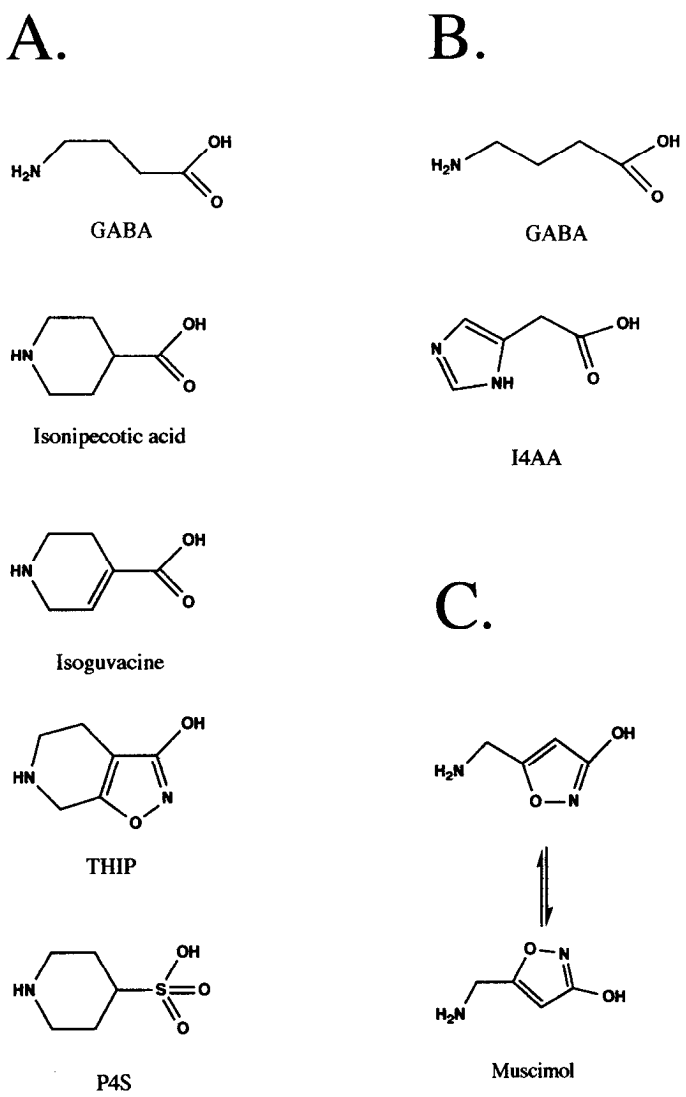

Figure 2. Molecular structures of GABA and several conformationally restricted analogs. *A*, GABA and its analogs in a partially folded conformation. Such a conformation is preferred by $GABA_A$ receptors, and all the compounds illustrated are potent $GABA_A$ receptor agonists. *B*, GABA and its analog (I4AA) in a fully extended conformation. I4AA is a weak agonist on $GABA_A$ receptors. *C*, Muscimol can exist in both a fully extended and partially folded conformation.

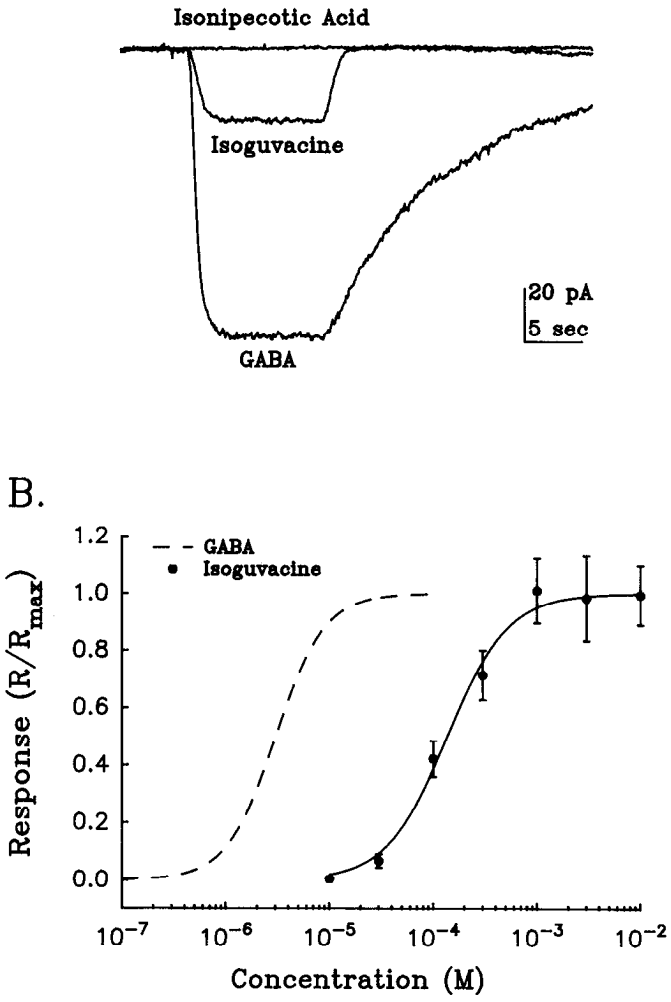

Figure 3. *A*, The effects of GABA (10 μM), isoguvacine (100 μM), and isonipecotic acid (100 μM) on an H4 horizontal cell. *B*, Dose–response curve for isoguvacine on H4 horizontal cells. Data were from seven cells; for each cell the responses are expressed as a fraction of the maximum response on that cell induced by 100 μM GABA, R_{max}. The *continuous curve* is a mathematical fit of the data points to the Hill equation with the parameters of $n = 1.48$, $EC_{50} = 137$ μM. The *dashed line* indicates the dose–response curve for GABA as shown in Figure 1*B*. The maximum response for isoguvacine (10 μM) was 100.1 ± 27.4 pA, a value close to the maximum response elicited by 100 μM GABA on the same group of cell (96.3 ± 17.2 pA).

= 301 μM. For a competitive antagonist, the IC_{50} value allows for an estimation of the inhibition constant (K_i), which was 85 μM for THIP (Eq. 3). That the inhibition by THIP is competitive is shown in Figure 4*B*. In the presence of 1 mM THIP, the GABA dose–response curve was shifted to the right on the abscissa without affecting the maximum GABA response. The amount shifted (from an EC_{50} of 3.03 μM in Ringer's to an EC_{50} of 38.6 μM in the presence of 1 mM THIP) can be used to calculate more precisely the inhibition constant, K_i, according to Equation 4. The calculated K_i for THIP was 85.2 μM, close to the K_i value estimated from the IC_{50} value.

P4S (piperidine-4-sulfonic acid), another specific and powerful $GABA_A$ receptor agonist, is a derivative of isonipecotic acid in which the carboxyl group is substituted by a sulfonate group (Krogsgaard-Larsen et al., 1980). When P4S was presented to H4 horizontal cells at concentrations less than 1 mM, no responses were observed. At concentrations greater than 1 mM, P4S activated small membrane currents. Thus, P4S acts as a partial agonist at the GABA site. Similar observations have been made on oocytes expressing GABA ρ1 subunits (Kusama et al., 1993). When P4S was coapplied with GABA, P4S reduced the GABA-induced responses (Fig. 4*A*). The calculated IC_{50} for P4S was 293 μM, which corresponds to an inhibition constant (K_i) of 82.8 μM. As was the case for THIP, 1 mM P4S shifted the GABA dose–response curve to the right on the abscissa without affecting the maximum response. That is, P4S also acts as competitive antagonist. The calculated inhibition constant was 80.9 μM for P4S.

Extended analogs. I4AA (imidazole-4-acetic acid) induced no responses in H4 horizontal cells at concentrations as high as 1 mM (data not shown). However, this agent acted as a powerful

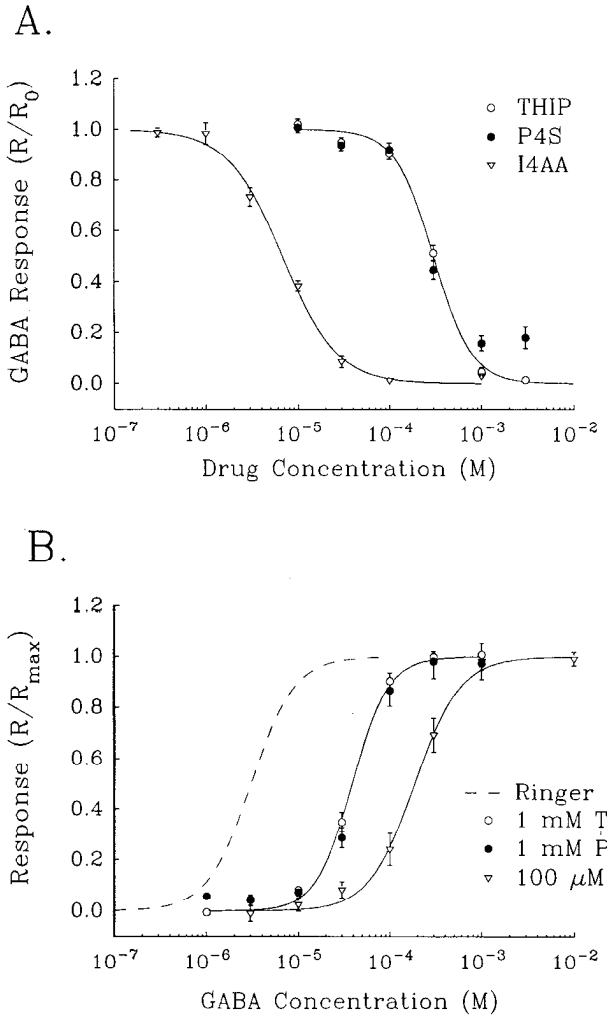

Figure 4. Effect of GABA analogs on GABA responses of H4 horizontal cells. *A*, Inhibition curves for THIP (six cells), P4S (six cells), and I4AA (five cells). For each cell the responses induced by 10 μM GABA in the presence of the agents are expressed as a fraction of the response induced by 10 μM GABA in Ringer's (R_0). The continuous curves are a mathematical fit of the data points to Equation 2, with n = 2.1 and IC_{50} = 301 μM for THIP and n = 1.46 and IC_{50} = 6.72 μM for I4AA. The calculated curve for P4S (not shown) is similar to the curve for THIP with parameters of n = 1.7 and IC_{50} = 293 μM. The inhibition constants (K_i) calculated from IC_{50} values are 85.0, 82.8, and 1.90 μM for THIP, P4S, and I4AA, respectively. *B*, Dose–response curves for GABA on H4 horizontal cells in the presence of 1 mM THIP (six cells), 1 mM P4S (five cells), and 100 μM I4AA (five cells). For each cell responses to 100 μM GABA in Ringer's solution were treated as R_{max}. The *continuous curves* are a mathematical fit of the data points to Equation 1 with parameters of n = 2.2 and EC_{50} = 38.6 μM for the GABA response in the presence of THIP, and n = 1.74 and EC_{50} = 186 μM in the presence of I4AA. The GABA response curve in the presence of P4S (not shown) has parameters of n = 2.17 and EC_{50} = 44.1 μM. For comparison, the GABA dose–response curve in Ringer's from Figure 1*A* is replotted as the *dashed line*. The inhibition constants calculated from EC_{50} values are 85.2, 80.9, and 1.67 μM for THIP, P4S, and I4AA, respectively. These data are presented as mean ± SE.

competitive antagonist on GABA-induced responses in H4 horizontal cells. Indeed, of all the analogs tested in this study, I4AA was the most potent. Responses induced by 10 μM GABA in the presence of I4AA are shown in Figure 4*A*. The continuous curve is a mathematical fit of the data to Equation 2 with parameters of n = 1.46 and IC_{50} = 6.72 μM. The calculated inhibition constant (K_i) was 1.92 μM. In the presence of 100 μM I4AA, the GABA dose–response curve was shifted to the right on the abscissa without affecting the maximum response (Fig. 4*B*). The amount shifted (from an EC_{50} of 3.03 μM in Ringer's to an EC_{50} of 186 μM in the presence of 100 μM I4AA) indicates an inhibition constant of 1.67 μM for I4AA.

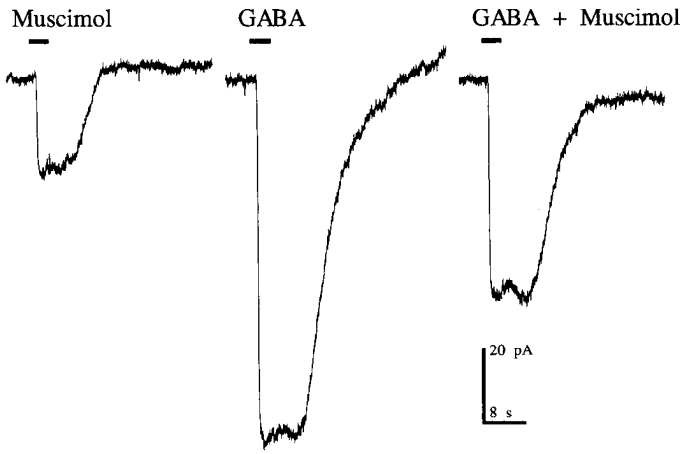

Figure 5. The responses of an H4 horizontal cell to 100 μM muscimol (*left*), 10 μM GABA (*center*), and the coapplication of 10 μM GABA and 100 μM muscimol (*right*). Note the response induced by coappling GABA and muscimol was smaller than the response induced by GABA alone, indicating an antagonistic effect of muscimol on the GABA response.

Analogs with mixed conformation. Muscimol is a potent $GABA_A$ receptor agonist. Although muscimol can also induce some response in H4 horizontal cells, it is less effective than is GABA. In addition, the maximum response to muscimol is only about one-third of the maximum GABA response regardless of concentration (see Fig. 4 in Qian and Dowling, 1993a). This implies that muscimol acts as partial agonist on H4 horizontal cells. Muscimol can also antagonize the GABA response as shown in Figure 5. Figure 5 (left) shows a response of a horizontal cell to 100 μM muscimol, which was about 36% of the response induced in that cell by 10 μM GABA (Fig. 5, center). When 100 μM muscimol was coapplied with 10 μM GABA, the response was only about 60% of the size of the response elicited by 10 μM GABA alone (Fig. 5, right). Similar inhibition of GABA responses by muscimol was observed in four other cells.

$GABA_A$ receptor antagonists

Bicuculline is the most common competitive $GABA_A$ receptor antagonist, and its effectiveness is often used as the criterion for the involvement of $GABA_A$ receptors in a response (Hill and Bowery, 1981). The GABA responses from H4 horizontal cells are almost completely resistant to bicuculline. Even with a concentration of 500 μM bicuculline, 96% of GABA responses remain (Qian and Dowling, 1993a). Hydrastine, a recently developed and potent competitive $GABA_A$ receptor antagonist (Huang and Johnston, 1990) also did not substantially block the GABA responses in H4 horizontal cells (Fig. 6*A*). When 100 μM of hydrastine was coapplied with 10 μM GABA, the responses elicited were decreased by less than 20% (0.19 ± 0.06, n = 4). A failure to block the GABA responses in H4 horizontal cells was also observed with SR-95531 (gabazine), another specific competitive $GABA_A$ receptor antagonist (Heaulme et al., 1986) (Fig. 6*B*). In the presence of 100 μM SR-95531, 74% of the control responses remained (0.74 ± 0.08, n = 4).

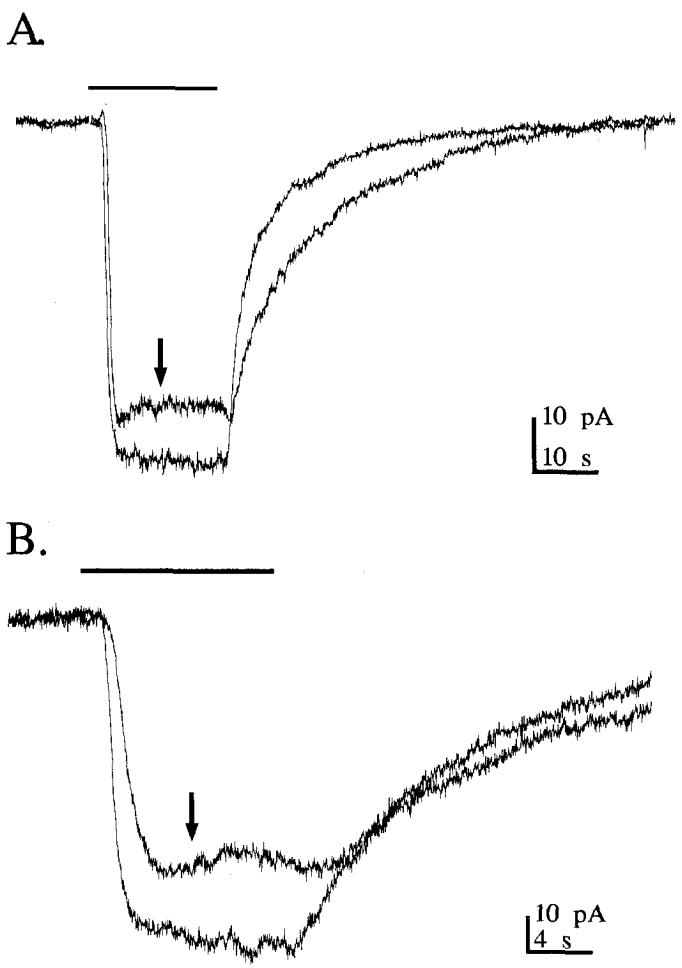

Figure 6. Effect of 100 μM hydrastine (*A*) and 100 μM SR-95531 (*B*) on the GABA responses of H4 horizontal cells. *Arrows* point to GABA responses obtained in the presence of the agent. The *bar* above the traces shows the duration of GABA application.

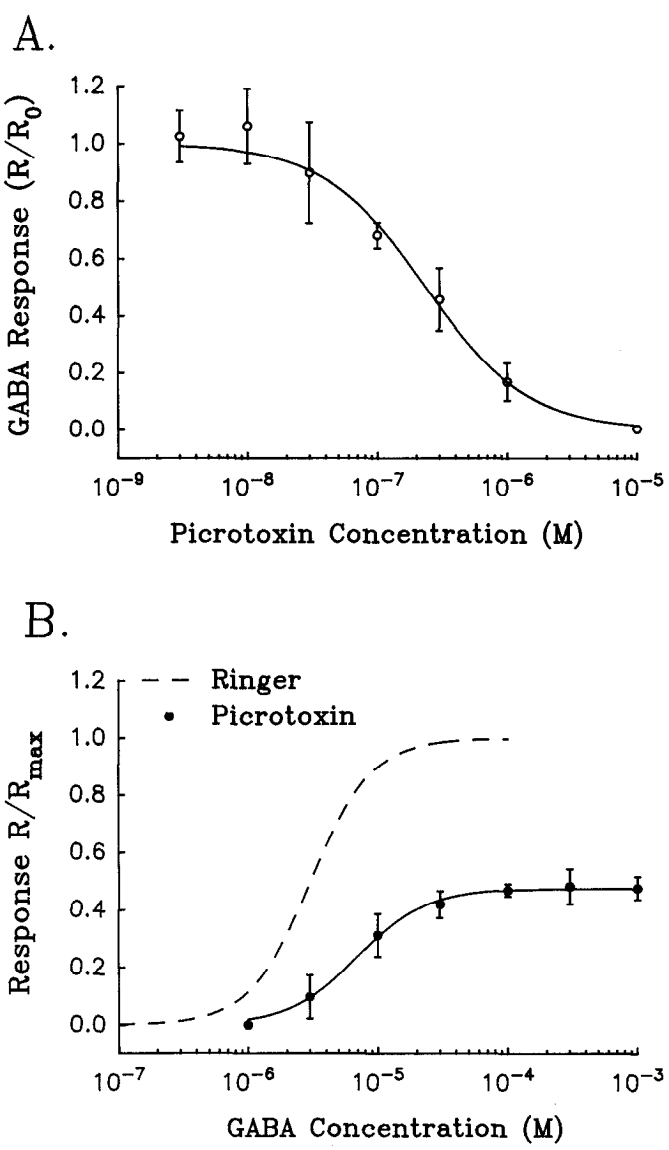

Figure 7. Effect of picrotoxin on GABA responses of H4 horizontal cells. *A,* Inhibition curve for picrotoxin. The continuous curve is a mathematical fit of data points to Equation 2 with parameters of n = 1.11 and IC_{50} = 237 nM. Data are from four cells. *B,* Dose–response curves of GABA on H4 horizontal cells in the presence of 1 μM picrotoxin. Data are from four cells. The dose–response curve for GABA is shifted to the right and is vertically compressed in the presence of picrotoxin, indicating that picrotoxin inhibits the responses by both competitive and noncompetitive mechanisms. The *continuous curve* is a mathematical fit of the data points to Equation 4 with parameters of n = 1.63 and EC_{50} = 6.97 μM. See Figure 4 for more details.

Picrotoxin is the most commonly used noncompetitive $GABA_A$ receptor antagonist. It behaves as a chloride channel blocker on these receptors, reducing the amplitude of $GABA_A$-mediated responses without affecting EC_{50} values (Akaike et al., 1985; Inoue and Akaike, 1988). The GABA responses of H4 horizontal cells were very sensitive to picrotoxin. An inhibition curve for picrotoxin on H4 horizontal cell responses is shown in Figure 7*A*. The data can be fitted by Equation 2 with an IC_{50} value of 237 μM, which is an order of magnitude lower than the IC_{50} value reported for typical $GABA_A$ receptors (Inoue and Akaike, 1988; Lehoullier and Ticku, 1989).

The inhibition by picrotoxin of GABA responses in H4 cells is both competitive and noncompetitive. Dose–response curves of the GABA response elicited in the absence and presence of 1 μM picrotoxin are shown in Figure 7*B*. In the presence of 1 μM of picrotoxin, about 20% of the GABA response remained (Fig. 7*A*). Twenty percent of the response in Figure 7*B* corresponds to a GABA concentration of 8 μM, a value very close to that used in the experiments shown in Figure 7*A* (i.e., 10 μM). The GABA dose–response curve was shifted to the right in the presence of picrotoxin, indicating the competitive nature of the effect; on the other hand, the maximum response was also reduced, indicating a noncompetitive component to the blockade. A similar phenomenon has been observed for picrotoxin on the GABA responses of oocytes expressing bovine retinal mRNA (Woodward et al., 1992).

TBPS (*tert*-butylbicyclophosphorothionate) is a potent $GABA_A$ receptor channel blocker that is thought to bind to the same site on $GABA_A$ receptors as picrotoxin (Akaike et al., 1985; Tehrani et al., 1986; Ticku 1986; Renterghem et al., 1987; Inoue and Akaike, 1988). The effects of TBPS on the GABA responses elicited from white perch H4 horizontal cells are shown in Figure 8. The responses are expressed as the fraction of the response induced by 10 μM GABA in Ringer's. No significant inhibition of the GABA-induced responses was observed in the presence of TBPS at concentrations as high as 10 μM. Even in the presence

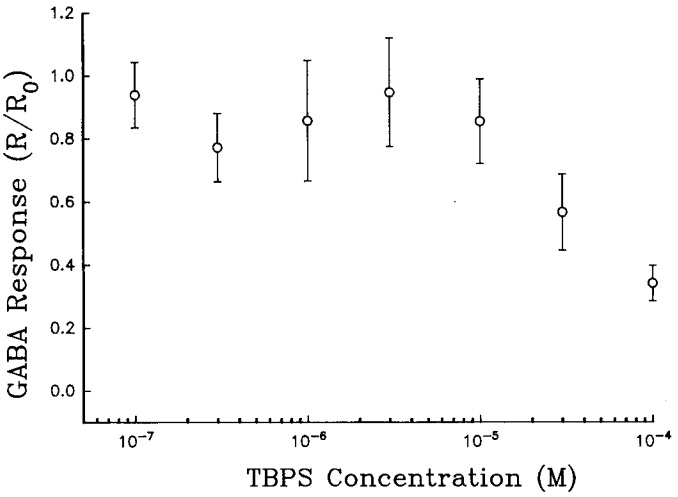

Figure 8. Effect of TBPS on GABA responses of H4 horizontal cells. Data were averaged from five cells. No significant reduction of the GABA response was observed in the presence of TBPS at concentrations as high as 10 μM. Even at 100 μM, 30% of the GABA response remained.

of 100 μM TBPS, about 30% of the GABA-induced response remained.

$GABA_B$ receptor agonists

Phosphate analogs of GABA act as $GABA_B$ receptor agonists. An example is 3-aminopropyl phosphonic acid (3-APA), which is a selective $GABA_B$ receptor agonist (Slaughter and Pan, 1992). When 3-APA was coapplied with GABA on H4 horizontal cells, it inhibited the GABA-induced responses (Fig. 9*A*). The IC_{50} for 3-APA was 165 μM, which corresponds to an inhibition constant of 47 μM. The inhibition by 3-APA on H4 horizontal cell GABA responses is also competitive, as shown in Figure 9*B*. In the presence of 500 μM 3-APA, the dose–response curve for GABA was shifted to the right without a change in the maximum response. The calculated inhibition constant for 3-APA was 43.1 μM.

Glycine receptor antagonists

Portions of the ρ1 subunit are as homologous to the subunits of glycine receptors as to the subunits of $GABA_A$ receptors (Cutting et al., 1991). Since H4 horizontal cells of perch possess glycine receptors (Zhou et al., 1993), an obvious question is whether the GABA responses observed here could be mediated through glycine receptors. To test this, strychnine, a competitive glycine receptor antagonist, was coapplied with GABA. Strychnine was relatively ineffective in blocking the GABA responses. In the presence of 500 μM strychnine, GABA responses elicited with 10 μM GABA were 60% of control responses (0.60 ± 0.11, $n = 4$).

Discussion

The GABA responses elicited from H4 horizontal cells of the white perch are clearly distinct from classic $GABA_A$ and $GABA_B$ receptor–mediated responses. On the other hand, the responses observed in H4 horizontal cells are very similar to the GABA responses obtained from oocytes expressing bovine retina mRNA and mRNA coding for the human ρ1 GABA channel subunit (Kusama et al., 1993; Woodward et al., 1993). Thus, we support the view that the receptors mediating the GABA responses in H4 horizontal cells are unique receptors and that they should

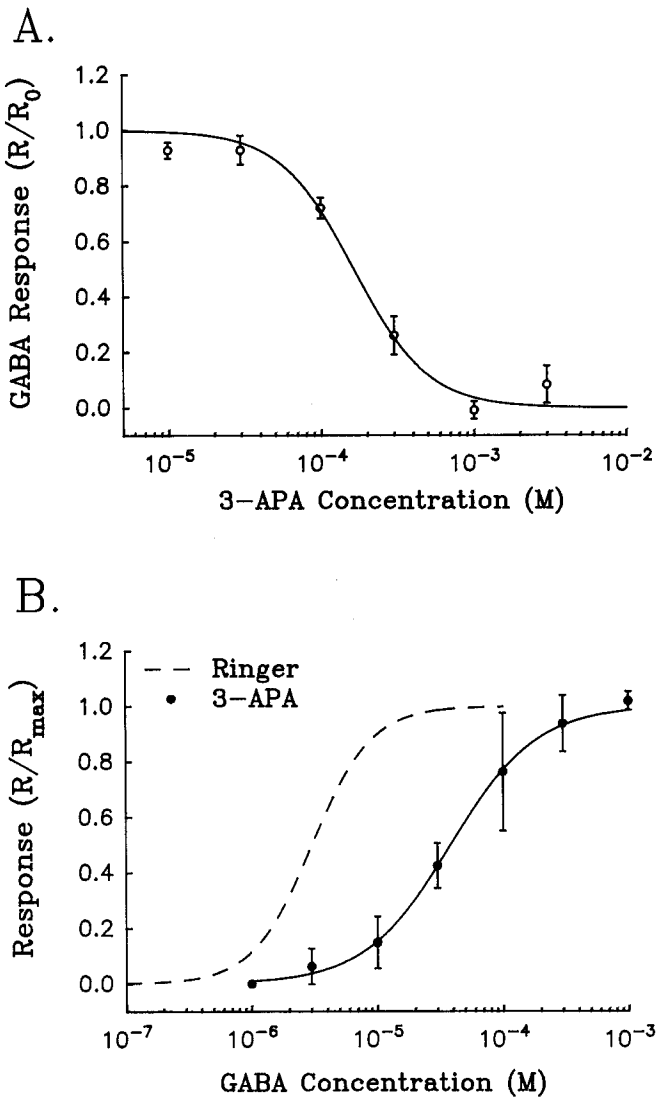

Figure 9. Effect of 3-APA, a selective $GABA_B$ agonist, on GABA responses of H4 horizontal cells. *A*, Inhibition curve for 3-APA. Data are from five cells. The continuous curve is a mathematical fit of the data points to Equation 2 with $n = 1.81$ and $IC_{50} = 165$ μM, and they indicate an inhibition constant (K_i) of 47 μM. *B*, Dose–response curves for GABA on H4 horizontal cells in the presence of 500 μM 3-APA. Data are from five cells. The *continuous curve* is a mathematical fit of the data points to Equation 1 with parameters of $n = 1.27$ and $EC_{50} = 38.2$ μM. The calculated inhibition constant is 43.1 μM. See Figure 4 for more details.

be termed $GABA_C$ receptors (Shimada et al., 1992; Woodward et al., 1993).

Because the GABA receptors observed in H4 horizontal cells are linked to a chloride channel, it might be argued that the receptors mediating these responses are a $GABA_A$ receptor subtype. However, the GABA responses elicited from H4 horizontal cells of the white perch differ physiologically and pharmacologically from classic $GABA_A$ receptor responses in every way examined so far. Physiologically, GABA responses from H4 horizontal cells are sustained, showing no sign of desensitization, and they have a slow time course of recovery. Pharmacologically, the GABA responses from H4 horizontal cells cannot be blocked by bicuculline, are more sensitive to GABA than are typical $GABA_A$ receptors, and they cannot be modulated by either barbiturates or benzodiazepines (Qian and Dowling,

1993a). Furthermore, the responses are less sensitive to muscimol than to GABA itself (Qian and Dowling, 1993a), and muscimol acts as a partial agonist on H4 horizontal cells. In addition, as we show here, none of the GABA analogs in a partially folded conformation (isoguvacine, isonipecotic acid, THIP, and P4S) potently activates the GABA responses on H4 horizontal cells. All of these compounds strongly activate $GABA_A$ receptors, but they either have no effect, act as partial agonists, or act as antagonists on the GABA receptors of H4 horizontal cells. On the other hand, I4AA, a GABA analog in a fully extended conformation, strongly inhibits the GABA responses on H4 horizontal cells. Indeed, the inhibition constant (K_i) of I4AA on $GABA_C$ receptors is comparable to the EC_{50} value of GABA on $GABA_C$ receptors, suggesting that the fully extended GABA conformation is preferred for $GABA_C$ receptor binding. Thus, our results indicate that a different conformation of GABA is required to interact with the receptors on H4 horizontal cells than with $GABA_A$ receptors. This notion is supported also by the observations that competitive antagonists of $GABA_A$ receptors, including bicuculline, hydrastine, and SR-95531, have little effect on the GABA responses of H4 horizontal cells. Furthermore, the H4 horizontal cell receptors are more sensitive to picrotoxin than are classic $GABA_A$ receptors, and picrotoxin acts as both a competitive and noncompetitive antagonist on these receptors. These receptors are also relatively insensitive to TBPS, another $GABA_A$ receptor chloride channel blocker. These latter observations indicate that the chloride channels linked to the GABA receptors on H4 horizontal cells also differ from the channels linked to classic $GABA_A$ receptors.

There is also molecular evidence that $GABA_C$ receptors are distinct from $GABA_A$ receptors. Although the $\rho 1$ subunit was initially thought to be a $GABA_A$ receptor subunit, portions of it are as homologous to glycine receptor subunits as to $GABA_A$ receptor subunits (Cutting et al., 1991). More importantly, the GABA responses of oocytes coexpressing both the $\rho 1$ subunits and individual $GABA_A$ subunits are indistinguishable from the responses of oocytes expressing the $\rho 1$ subunit alone, indicating that the $GABA_A$ and $\rho 1$ subunits do not aggregate (Shimada et al., 1992). In oocytes expressing total retinal mRNA, two distinct types of GABA responses were observed, a classic $GABA_A$ receptor response and a $GABA_C$ receptor–mediated response (Polenzani et al., 1991). This also indicates that $GABA_A$ and $GABA_C$ subunits aggregate independently of one another. $GABA_A$ subunits readily aggregate in oocytes to form heteroligomeric receptors with the properties of classic $GABA_A$ receptors (Seeburg et al., 1990; Burt and Kamatchi, 1991), whereas $\rho 1$ subunits aggregate in oocytes to form homooligomeric $GABA_C$ receptors (Shimada et al., 1992). The similarity of the GABA responses of H4 horizontal cells to those of oocytes expressing only the $\rho 1$ subunit suggests that the functional receptors on the H4 horizontal cells could be homooligomeric.

Woodward et al. (1993) have recently proposed that the terminology ρ-like GABA receptors be used for these receptors, rather than $GABA_C$ receptors. We do not feel that this is appropriate terminology. As noted above, the ρ subunit was first thought to be a $GABA_A$ subunit and is often included in listings of $GABA_A$ receptor subunits (Burt and Kamatchi, 1991; Cutting et al., 1991); thus, the terminology "ρ-like GABA receptor" suggests that these receptors are a subtype of $GABA_A$ receptor. We prefer the term $GABA_C$ receptor, which was first introduced by Johnston (Johnston et al., 1986), to describe receptor-mediated responses that are bicuculline- and baclofen-insensitive, and that are inhibited by high concentrations of isoguvacine (Drew and Johnston, 1992). Such properties are very similar to the GABA responses from H4 horizontal cells and the responses of oocytes expressing bovine retinal mRNA and the $\rho 1$ subunit. The one difference between the results of Johnston et al. and the more recent results involves the effectiveness of *cis*-amino crotonic acid (CACA), a GABA analog conformationally constrained around one of its C–C bonds. Whereas the binding sites described by Johnston et al. were more sensitive to CACA than to its more extended *trans* isomer (TACA, *trans*-aminocrotonic acid), the GABA responses elicited from the perch horizontal cells and from oocytes expressing $\rho 1$ subunits or retinal mRNA are more sensitive to TACA than to CACA (Kusama et al., 1993; Woodward et al., 1993; H. Qian and J. E. Dowling, unpublished observation). This may indicate that $GABA_C$ receptors are somewhat heterogeneous, similar to $GABA_A$ and $GABA_B$ receptors. Indeed, a second ρ subunit ($\rho 2$) has now been described and more may exist (Cutting et al., 1992). Also, it should be noted that bicuculline-resistant GABA responses have been reported in several invertebrate preparations. These receptors may be variant members of the $GABA_C$ receptor family. It is also possible that the low potency of CACA reflects a discrepancy between receptor binding studies and physiological studies.

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