Multiple GABA Receptor Subtypes Mediate Inhibition of Calcium Influx at Rat Retinal Bipolar Cell Terminals

Zhuo-Hua Pan¹ and Stuart A. Lipton²

¹Department of Neurology, Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115 and

²Department of Neurology, Children's Hospital, Beth Israel Hospital, Brigham and Women's Hospital, Massachusetts General Hospital, and Program in Neuroscience, Harvard Medical School, Boston, Massachusetts 02115

Inhibitory effects of GABA on K⁺-evoked Ca²⁺ influx into rat retinal bipolar cell terminals were studied using calcium imaging methods. Application of high K⁺ evokes a sustained, reversible increase in [Ca²⁺]_i at bipolar cell terminals, which occurs mainly via dihydropyridine-sensitive (L-type) Ca²⁺ channels. There are at least two GABA receptor subtypes coexisting at bipolar cell terminals: a conventional GABA_A receptor and a bicuculline/baclofen-insensitive GABA receptor. Activation of either GABA receptor inhibited the K⁺-evoked Ca²⁺ response. However, these two GABA receptor subtypes have distinct properties. GABA_A receptors suppress the Ca²⁺ response only at relatively high concentrations of agonist, and with fast kinetics and a narrow dynamic range. In contrast, the bicuculline/baclofen-insensitive GABA receptors produce inhibition on the Ca²⁺ response at a much lower concentration of agonist, and with slow onset and a wider dynamic range. The pharmacologic profile of the bicuculline/baclofen-insensitive GABA receptor at bipolar cell terminals is most similar to the GABA_C receptor reported by Feigenspan et al. (1993). Unlike the GABA_C receptors described in other species, it is extremely insensitive to picrotoxin. Therefore, it may be appropriate to refer to this receptor as a picrotoxin-insensitive GABA_C receptor. 3-Aminopropyl(methyl)phosphonic acid (3-APMPA) and 3-aminopropylphosphonic (3-APA), two phosphate analogs of GABA, selectively antagonize the picrotoxin-insensitive GABA_C receptors but not the GABA_A receptors in this system. These results imply a functional role for multiple GABA receptors in regulating synaptic transmission at bipolar cell terminals.

[Key words: GABA, GABA receptors, calcium, 3-aminopropyl(methyl)phosphonic acid, synaptic transmission, bipolar cells, retina, rat]

GABA is one of the major inhibitory neurotransmitters in the CNS in general and in the retina in particular (Olsen and Venter, 1986; Massey and Redburn, 1987; Tauck et al., 1988; Sivilotti and Nistri, 1991; Slaughter and Pan, 1992). There is abundant evidence that the terminals of retinal bipolar cells are highly

sensitive to GABA (Tachibana and Kaneko, 1987; Karschin and Wässle, 1990; Suzuki et al., 1990; Yeh et al., 1990; Heidelberger and Matthews, 1991). It is speculated that the input of GABAergic amacrine cells to bipolar cell terminals creates negative feedback to generate transient responses in the inner retina (Tachibana and Kaneko, 1987; Maguire et al., 1989).

Recently, a novel bicuculline/baclofen-insensitive GABA receptor has been reported on tectal neurones of the frog (Nistri and Sivilotti, 1985; Sivilotti and Nistri, 1989) and retinal neurons in several species (Feigenspan et al., 1993; Qian and Dowling, 1993; Dong et al., 1994; Lukasiewicz et al., 1994; Matthews et al., 1994; Zhang and Slaughter, 1994). The newly cloned GABA_B subunits and GABA_B-like responses encoded by bovine retinal mRNA, expressed in *Xenopus* oocytes, display similar properties (Cutting et al., 1991; Polenzani et al., 1991; Shimada et al., 1992; Kusama et al., 1993b). This novel GABA receptor is commonly referred to as the GABA_C receptor (Johnston, 1986; Shimada et al., 1992). Although a similar GABA receptor has been found in several species, the GABA_C receptor of rat bipolar cells has been reported to be insensitive to picrotoxin (Feigenspan et al., 1993). The implication of this discrepancy is not clear, since the pharmacologic properties and molecular identities of the putative GABA_C receptor are still not fully understood.

Very few studies have examined selective antagonists for this newly described GABA receptor or family of receptors. Several phosphate analogs of GABA, which are the GABA_B receptor agonists or antagonists (Slaughter and Pan, 1992), and imidazole-4-acetic acid (I4AA), a GABA analog with an extended conformation (Kusama et al., 1993; Qian and Dowling, 1994), have been reported to display antagonist properties to the bicuculline/baclofen-insensitive GABA receptors expressed in *Xenopus* oocytes (Kusama et al., 1993a; Woodward et al., 1993). I4AA has also been reported to be an effective antagonist of the GABA_C receptor in fish retinal horizontal cells (Qian and Dowling, 1994). However, the properties of these compounds have not been studied on the picrotoxin-insensitive type of GABA_C receptors found in the rat retinal neurons.

Recent studies have shown that GABA can act at multiple subtypes of GABA receptors on bipolar cells. Both GABA_A and GABA_C receptors have been reported in retinal bipolar cells of goldfish, rat, tiger salamander, and white perch (Tachibana and Kaneko, 1987; Feigenspan et al., 1993; Qian and Dowling, 1993b; Lukasiewicz et al., 1994; Matthews et al., 1994). Previously, a baclofen-sensitive GABA_B receptor has been reported in tiger salamander bipolar cell terminals (Maguire et al., 1989).

Received Aug. 24, 1994; revised Oct. 13, 1994; accepted Oct. 24, 1994.

We thank Dr. Malcolm M. Slaughter for helpful discussions and valuable comments on the manuscript. This work was supported in part by a Scholar Award from the American Foundation for AIDS Research (to Z.-H.P.) and by NIH Grant R01 EY05477 (to S.A.L.).

Correspondence should be addressed to Dr. Zhuo-Hua Pan at the above address.

Copyright © 1995 Society for Neuroscience 0270-6474/95/152668-12\$05.00/0

In goldfish bipolar cell terminals, a baclofen-insensitive GABA_B-like receptor has also been reported (Heidelberger and Matthews, 1991; Matthews et al., 1994). The common effect of all of these GABA receptors is, in one way or another, to regulate the Ca²⁺ response of the terminal. Although the inhibitory effect of GABA on Ca²⁺ influx into giant goldfish bipolar terminals has been directly visualized with calcium imaging methods (Heidelberger and Matthews, 1991; Matthews et al., 1994), a detailed picture of the interaction of multiple GABA receptor subtypes in regulating Ca²⁺ responses at bipolar cell terminals, especially in mammalian species, is not clear. Furthermore, the functional significance of the coexistence of GABA receptor subtypes at bipolar cell terminals remains unknown.

In this study, by using calcium imaging, we demonstrated the colocalization of multiple GABA receptor subtypes at rat bipolar cell terminals. Particularly, we further characterized the pharmacologic properties of the GABA_C receptor and the dynamic course of inhibition resulting from multiple GABA receptor subtypes at rat bipolar cell terminals. Previous studies have mainly focused on the responses recorded from the bipolar cell soma. An advantage here is that the responses of synaptic terminals could be directly visualized.

A preliminary report of part of this work has appeared (Pan and Lipton, 1994b).

Materials and Methods

Bipolar cell isolation. Bipolar cells were dissociated from 3–4 week postnatal Long-Evans rats. The dissociation and culture methods are similar to those previously published, with minor modifications (Leifer et al., 1984; Lipton and Tauck, 1987). In brief, after decapitation of the animal, both retinas were removed and placed in a saline based upon Hanks' balance salts (in mM): NaCl, 137; NaHCO₃, 1; Na₂HPO₄, 0.34; KCl, 5.36; KH₂PO₄, 0.44; CaCl₂, 1.25; MgSO₄, 0.5; MgCl₂, 0.5; HEPES-NaOH, 5; glucose, 22.2; with phenol red, 0.001% v/v; adjusted to pH 7.2 with 0.3 N NaOH. The retinas were incubated for 50 min at 37°C in 10 ml of an enzyme solution that consisted of the saline described above, supplemented with 0.2 mg/ml DL-cysteine, 0.2 mg/ml bovine serum albumin, and 3.5 U/ml papain, adjusted to pH 7.2 with 0.3 N NaOH. Following three rinses in saline, the retinas were mechanically dissociated by gentle trituration with a glass pipette. The resulting cell suspension was plated onto poly-L-lysine-coated glass coverslips. The culture medium was comprised of Eagle's essential medium supplemented with 2 mM glutamine, 1 µg/ml gentamicin, 16 mM glucose, and 5% rat serum. Cell cultures were incubated at 37°C in a 5% CO₂/95% O₂ humidified atmosphere for about 1 hr before dye loading.

Dye loading. Prior to dye loading, the culture medium was replaced with Hanks' solution. Then, cells were loaded with the membrane-permeable fluorescence dye fluo-3/AM (Molecular Probes, OR) at a concentration of 20 µM, supplemented with 0.0025% pluronic acid, in darkness at room temperature for 1 hr (Kao et al., 1989; Pan and Lipton, 1994a). After dye loading, the cells were washed with Hanks' solution to remove the remaining dye. Cells were kept in darkness for another hour. Before fluorescence measurements, a coverslip of cells was transferred to a glass-bottomed chamber on the stage of a confocal microscope. The cells were continuously superfused at a rate of about 1 ml/min with a recording solution. The recording solution is the same as the Hanks' solution described above except that it contains 2.5 mM CaCl₂.

Bipolar cell identification. The intact bipolar cells could be easily identified based on their characteristic morphology (Tachibana and Kaneko, 1987; Karschin and Wässle, 1990; Yeh et al., 1990). We did not make efforts to identify the subtype of bipolar cells, but we preferentially selected bipolar cells with long axons. Based on previous reports, virtually all of these dissociated bipolar cells are rod bipolar cells (Karschin and Wässle, 1990).

High potassium application and drug delivery. Depolarization was evoked by high K⁺ saline, formulated by replacement of equimolar Na⁺ in the recording solution. A number of K⁺ concentrations were tested to determine the proper K⁺ to evoke a strong Ca²⁺ signal but not to

saturate the fluorescence Ca²⁺ indicator. Under our condition, 80 mM K⁺ evoked an equivalent or sometimes slightly smaller fluorescence signal than that evoked by 60 mM K⁺. However, 40 mM K⁺ evoked a consistently smaller fluorescence signal than 60 mM K⁺ (85% ± 3%, mean ± SEM, *n* = 7). In order to assure that the Ca²⁺ indicator was not saturated by [Ca²⁺]_i, 40 mM K⁺ was therefore used to evoke Ca²⁺ responses throughout this work. Pharmacological agents were mixed with high K⁺ saline and applied by pneumatic ("puffer") pipettes placed about 30–40 µm away from the bipolar cell terminals, unless otherwise specified.

Fluorescence measurement. Fluorescence measurements were performed at room temperature on a confocal laser-scanning microscope (NORAN, WI) coupled to an imaging system (Universal Imaging, PA). The video scan module (Odyssey) was mounted on an upright Nikon microscope fitted with a 40×, 0.75 NA water-immersion objective. Optical excitation was accomplished using the 488 nm line of an argon laser. The emitted fluorescence passed through a 515 nm primary barrier filter before it reached the photomultiplier tube. The laser intensity was minimized to prevent dye bleaching during the course of the experiments. During measurements, the fluorescence images were acquired in real-time mode (30 frames/sec), and the fluorescence data at a defined bipolar cell terminal were stored and later exported to a SIGMAPLOT graphics program. In some cases, the real-time video images were directly recorded onto an optical memory disc recorder (OMDR, Panasonic) for later analysis.

Data analysis. Since fluo-3 is a single-wavelength chromophore, the fluorescence is a function of the [Ca²⁺]_i as well as the dye concentration. However, for a fixed region, the change of fluorescence directly reflects the change of [Ca²⁺]_i. Therefore, data are not calibrated to absolute values of [Ca²⁺]_i, but are displayed as a ratio of fluorescence change. For the purpose of clarity, we will use the term "calcium" instead of "fluorescence" throughout the paper. A total of 826 bipolar cell terminals were studied in this report. Each result reported in this paper was based on the observations obtained from at least four bipolar cell terminals.

Chemicals. 3-Aminopropylphosphonic acid (3-APPA), *trans*-, and *cis*-4-aminocrotonic acid (TACA and CACA) were purchased from Tocris Neuramin (Bristol, UK); 3-aminopropyl(methyl)phosphonic acid (3-APMPA), 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), muscimol, SR95531, phaclofen, hydroxy-saclofen, and nimodipine from Research Biochemicals Inc. (Natick, MA); GABA, (–)-bicuculline methobromide, picrotoxinin, strychnine, imidazole-4-acetic acid (I4AA), piperidine-4-sulfonic acid (P4S), baclofen, and 3-aminopropylphosphonic acid (3-APA) from Sigma (St. Louis, MO).

Results

Characterization of calcium responses at bipolar cell terminals

As shown in Figure 1, *A* and *B*, a sustained increase in [Ca²⁺]_i at bipolar cell terminals was elicited by applying high K⁺ (40 mM). There was no significant fading of the Ca²⁺ response during >20 sec of continuous measurement. After removing the high K⁺, the [Ca²⁺]_i rapidly recovered to the basal level, demonstrating the Ca²⁺ buffering capacity of bipolar cell terminals (Fig. 1*B*).

To test whether the increase in [Ca²⁺]_i evoked by high K⁺ was due to influx of extracellular Ca²⁺, high K⁺ was applied in Ca²⁺-free saline containing 5 mM EGTA. Under these conditions, no increase in [Ca²⁺]_i was observed (data not illustrated). Thus, influx of extracellular Ca²⁺ is essential for a depolarization-evoked increase in [Ca²⁺]_i.

To determine the subtype of Ca²⁺ channel responsible for the sustained Ca²⁺ response in bipolar cell terminals, we used nimodipine, a specific L-type Ca²⁺ channel blocker. The Ca²⁺ response was substantially suppressed when 4 µM nimodipine was coapplied with high K⁺ in the pneumatic pipette and included in the bath solution (Fig. 1*C*). Therefore, most of the Ca²⁺ influx evoked by high K⁺ appears to occur via an L-type Ca²⁺ channel. Further characterization of the Ca²⁺ channels at rat bipolar cell terminals was beyond the scope of this work.

Figure 1. Potassium-evoked increase in $[Ca^{2+}]_i$ at bipolar cell terminals. **A**, Fluorescence, pseudocolor images of a representative bipolar cell before and during application of 40 mM K^+ . The horizontal scale bar represents 5 μm . The fluorescence (left vertical bar) is an arbitrary 256 point gray scale converted to pseudocolor. **B**, Time course of fluorescence measured at another bipolar cell terminal. High K^+ (40 mM and the same in all figures) application is indicated by a dark bar above the trace. The vertical axis is the fluorescence normalized to that of the basal level. **C**, Nimodipine (4 μM) suppressed the majority of the Ca^{2+} response. In this case, 4 μM nimodipine was also included in the bath solution.

Inhibitory effect of GABA on calcium influx at bipolar cell terminals involves at least two components

Application of GABA itself at concentrations of 1–10 μM did not produce any increase in $[Ca^{2+}]_i$ at bipolar cell terminals (data not shown). When high K^+ and GABA were coapplied, as shown in Figure 2, GABA inhibited the K^+ -evoked Ca^{2+} response in a dose-dependent fashion. GABA produced a delayed, sustained inhibition on the Ca^{2+} response at submicromolar concentrations (Fig. 2*A,B*). GABA at concentrations of 1 μM or less, and usually at 2 μM , did not significantly suppress the initial peak of the Ca^{2+} response, but rather produced a delayed, sustained inhibition (Fig. 2*A–D*). This is demonstrated by comparing the Ca^{2+} responses evoked by high K^+ in the presence of 1 or 2 μM GABA with those evoked by high K^+ alone at the same terminals, as shown in Figure 2, *C* and *D*. In addition, GABA at a concentration of 1–2 μM , but not at submicromolar concentrations, frequently reduced the rate of rise of the Ca^{2+} response (Fig. 2*A–D*). Bicuculline, however, prevented this effect (see Fig. 3*A,B*). GABA at 5 μM or higher concentrations always completely suppressed the Ca^{2+} response (Fig. 2*E*). Bicuculline (100 μM) partially eliminated the suppression of the early part of the Ca^{2+} increase produced by 5 μM GABA, but did not block the delayed, sustained suppression (Fig. 2*F*). These results indicate that the suppression of the initial increase in $[Ca^{2+}]_i$ was, at least partially, due to the activation of bicuculline-sensitive GABA receptors.

The delayed, sustained inhibitory effect of GABA at a concentration of 1 μM was not significantly affected by bicuculline (100 μM), as demonstrated by measuring Ca^{2+} responses at the same terminals in the presence and absence of bicuculline (Fig. 3*A,B*). This indicates that GABA at ≤ 1 μM does not significantly activate GABA_A receptors during its delayed inhibition of Ca^{2+} response. However, bicuculline did increase the initial rise time of the Ca^{2+} response (Fig. 3*A,B*), consistent with the notion that the initial phase of GABA's action was mediated by GABA_A receptors, as mentioned above. Furthermore, the inhibitory effect of GABA at 1 μM concentration was not significantly affected by coapplication or bath application of 100 μM bicuculline plus 200 μM picrotoxinin (Fig. 3*C*). SR95531 (10 μM), a more potent GABA_A receptor antagonist, and strychnine (1 μM), a glycine receptor antagonist, did not block the inhibitory effect of GABA (Fig. 3*D,E*). Baclofen (100 μM) did not mimic GABA's inhibitory effect (Fig. 3*F*), and GABA_B receptor antagonists, such as phaclofen (100 μM) and hydroxy-saclofen (100 μM), did not block the action of GABA (data not illustrated).

Therefore, both conventional GABA_A and bicuculline/baclofen/picrotoxin-insensitive GABA receptors are involved in the inhibition of the Ca^{2+} response at rat bipolar cell terminals. These results are consistent with the earlier demonstration of a picrotoxin-resistant GABA_C receptor in recordings from rat bipolar cell somas (Feigenspan et al., 1993).

The picrotoxin-resistant property of this receptor on rat bipolar cells is clearly different from that of GABA_C receptors reported in retinal neurons of other species. Therefore, we tested for a picrotoxin-sensitive but bicuculline-insensitive component. We compared the time course of K^+ -evoked Ca^{2+} responses at a number of GABA concentrations in the presence of bicuculline (100 μM) with those elicited in the presence of bicuculline (100 μM) and picrotoxinin (200 μM). At relatively high GABA concentrations, for example 5 μM as shown in Figure 4, *A* and *B*,

Figure 2. Dose-dependent GABA suppression of the K^+ -evoked Ca^{2+} response and demonstration of the involvement of multiple GABA receptors. GABA was coapplied with high K^+ . *A–D*, GABA at concentrations of 1 or 2 μM did not significantly suppress the initial peak of Ca^{2+} response but produced a delayed, sustained inhibition and slightly reduced the rate of initial Ca^{2+} rise. *E*, Five micromolar GABA totally suppressed the Ca^{2+} response. *F*, When 100 μM bicuculline was coapplied with 5 μM GABA and high K^+ , an initial Ca^{2+} increase was observed. In *C–F*, a second Ca^{2+} response evoked by high K^+ alone was equivalent to the control (Fig. 1). These control Ca^{2+} responses were measured at the same terminal about 1 min after the initial measurements.

200 μM picrotoxinin did not show any significant effect on the time course of the Ca^{2+} response. At submicromolar GABA concentrations, for example 0.2 and 0.5 μM , 200 μM picrotoxinin partially blocked GABA's inhibitory effect (Fig. 4*C–F*). However, our data cannot resolve whether picrotoxinin blocks an additional component of delayed GABA inhibition or represents a weak antagonist for a single delayed component. Feigenspan et al. (1993) reported that picrotoxin blocks only a small percentage of the GABA current in rat bipolar cells. Taken together, these results indicate that the bicuculline/baclofen-insensitive GABA receptor in rat bipolar cell terminals is extremely picrotoxinin insensitive.

GABA_C receptor antagonists

We found that micromolar 3-APMPA, a potent GABA_B agonist, was able to block the inhibition of Ca^{2+} responses mediated by

the GABA_C receptor. 3-APMPA by itself, at concentrations as high as 5 mM, did not inhibit K^+ -evoked Ca^{2+} responses (Fig. 5*A*), but the delayed, sustained inhibitory effect produced by 1 μM GABA was completely blocked by 200 μM 3-APMPA (although inhibition of the initial Ca^{2+} rise was still present) (Fig. 5*B*). This finding is consistent with our observations that GABA at this concentration mainly activates GABA_C receptors, and the only visible inhibition due to the activation of the GABA_A receptor was on the very initial Ca^{2+} rise (compare Fig. 3*A,B*). As shown in Figure 5*C*, the inhibitory effect of GABA at higher concentrations (5 μM), which also activate GABA_A receptors (compare Fig. 2*E,F*), was not blocked by 200 μM 3-APMPA. Consistent with this interpretation, a combination of 100 μM bicuculline and 200 μM 3-APMPA totally blocked the inhibitory effect of high concentrations of GABA (10 μM) on the Ca^{2+} response (Fig. 5*D*).

Figure 3. Demonstration that the delayed, sustained inhibitory effect of 1 μ M GABA was not significantly affected by 100 μ M bicuculline (*A*, *B*), 100 μ M bicuculline plus 200 μ M picrotoxinin (*C*), 10 μ M SR95531 (*D*), or 1 μ M strychnine (*E*). One hundred micromolar baclofen did not mimic the inhibitory effect produced by GABA (*F*). However, the initial rate of rise of $[Ca^{2+}]_i$ was faster than that in the absence of bicuculline (compare *A* and *B*). In *A* and *B*, the measurements were performed at the same bipolar cell terminals.

To confirm further that 3-APMPA is a GABA_C, but not GABA_A, antagonist in our preparation, we tested the effects of 3-APMPA on two other putative GABA_A agonists, THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) and P4S (piperidine-4-sulfonic acid). These two GABA analogs have been reported to display agonist properties only at GABA_A receptors (Kusama et al., 1993a; Woodward et al., 1993; Qian and Dowling, 1994). Consistent with these reports, the inhibitory effects of THIP (100 μ M) and P4S (100 μ M) were blocked by bicuculline (100 μ M) (Fig. 6*A–D*). However, 3-APMPA, at concentrations as high as 500 μ M, did not block this inhibition (Fig. 6*E,F*).

We also tested 3-APA (3-aminopropylphosphonic acid) and 3-APPA (3-aminopropylphosphinic acid), two other phosphate

analogues of GABA, and I4AA (imidazole-4-acetic acid), a GABA analogue with an extended confirmation. We found that 3-APA by itself at a concentration of 500 μ M did not show any inhibition on the Ca^{2+} response (data not shown). Like 3-APMPA, 3-APA selectively blocked the GABA_C but not the GABA_A effect on Ca^{2+} responses, although it was less potent than 3-APMPA (data not illustrated). In addition, 3-APPA (200 μ M) and I4AA (100 μ M) displayed antagonistic effects on the GABA_C receptor in our preparation (data not shown). However, both of these drugs are also GABA_A receptor agonists at these concentrations. Therefore, they had to be administered with GABA_A antagonists to enable us to observe their inhibition on the GABA_C receptor in our preparation (data not illustrated).

Figure 4. High concentrations of picrotoxinin only slightly blocked the inhibitory effect produced by submicromolar concentrations of GABA. The same bipolar cell terminals were tested in the presence of 100 μM bicuculline (*A, C, E*) versus 100 μM bicuculline plus 200 μM picrotoxinin (*B, D, F*) at each GABA concentration. Bicuculline or bicuculline plus picrotoxinin were coapplied with GABA and high K⁺. Picrotoxinin (200 μM) did not significantly block the inhibitory effect produced by 5 μM GABA (*A, B*), and only slightly blocked the inhibitory effect by 0.2 μM and 0.5 μM GABA (*C–F*).

GABA_C receptor agonists

A number of GABA analogs, such as TACA (*trans*-aminocrotonic acid), CACA (*cis*-aminocrotonic acid), and muscimol, have been reported to activate the GABA_C receptor (Feigenspan, 1993; Qian and Dowling, 1993; Dong et al., 1994; Lukasiewicz et al., 1994; Matthew et al., 1994; Zhang and Slaughter, 1994). Consistent with these reports, we found that TACA exhibited a potency similar to GABA (Fig. 7*A*). Muscimol was also effective but less potent than GABA. As shown in Figure 7, *B* and *C*, muscimol at a concentration of 5 μM , but not 2 μM , mimicked the effect of submicromolar or low micromolar GABA (Fig. 7*B–D*). CACA had a similar effect but required much higher concentrations. In the presence of 100 μM bicuculline, 100 μM CACA produced incomplete inhibition of slow onset (Fig. 7*D*). The concentration of CACA had to be raised to 200 μM to totally suppress the Ca²⁺ response (Fig. 7*E*). Furthermore, the

effect of 200 μM CACA was totally blocked by 200 μM 3-APMPA (Fig. 7*F*), indicating that CACA at this concentration was selective for the GABA_C receptor. However, we found that the effect of 500 μM CACA was not blocked by 3-APMPA (500 μM) but was inhibited by a combination of 3-APMPA (500 μM) and bicuculline (100 μM) (Fig. 7*G,H*). This finding can be best rationalized by the notion that at such a high concentration, our preparation of CACA activates both GABA_C and GABA_A receptors.

Distinct effects of GABA_A versus GABA_C receptors on calcium responses

The selective antagonism of 3-APMPA and 3-APA on the GABA_C receptor made it possible to separate the effects of the GABA_A receptor from that of the GABA_C receptor, and therefore to compare the time courses of their inhibitory effects on Ca²⁺

Figure 5. 3-APMPA selectively blocks the GABA_C receptor. *A*, 3-APMPA (5 mM) did not inhibit the Ca²⁺ response to K⁺. *B*, 3-APMPA (200 μM) completely blocked the delayed, sustained inhibition of the Ca²⁺ response by 1 μM GABA (but did not prevent the slight reduction in rise time of the Ca²⁺ response). *C*, 3-APMPA (200 μM) did not block the inhibitory effect by 5 μM GABA. *D*, The combination of 200 μM 3-APMPA and 100 μM bicuculline completely blocked the inhibitory effect by 10 μM GABA.

responses. Paired measurements of K⁺-evoked Ca²⁺ responses in the presence of bicuculline/picrotoxinin versus 3-APMPA were performed on the same bipolar cell terminal for a range of GABA concentrations (Fig. 8). Several distinct properties of GABA_A and the GABA_C receptors were observed. First, an inhibitory effect on the GABA_C receptor was observed at GABA concentrations as low as 0.2 μM (Fig. 4E). However, GABA concentrations as high as 20 μM were needed to fully suppress the Ca²⁺ response by activating this receptor (Fig. 8G). In contrast, the inhibitory effect of the GABA_A receptor was virtually absent with 1–2 μM GABA (Fig. 8B, except for a slight reduction in the initial rise time of the Ca²⁺ response; compare Figs. 3A,B; 5B). Although GABA is more potent at the GABA_C receptor, the onset of the inhibition is slower. As shown in Figure 8E, even at concentrations of 10 μM, there was still an initial Ca²⁺ response. This contrasts with the effect of the GABA_A receptor, where ≥5 μM GABA always completely suppressed the early part of Ca²⁺ response (Fig. 8D). Finally, when the GABA_C component was blocked by 3-APMPA, the Ca²⁺ response in the presence of relatively low concentrations of GABA (5–10 μM) usually displayed a prominent rebound. The recovery of Ca²⁺ responses is not likely due to GABA_A receptor desensitization. The reason is that GABA at higher concentrations (20 μM) was found to eliminate this recovery (Fig. 8H), even when millimolar concentrations of 3-APMPA were used (data not shown). The latter makes incomplete blockade of GABA_C receptors unlikely.

Discussion

In this study, using a high-resolution confocal imaging system, we demonstrate the inhibitory effect of GABA on K⁺-evoked Ca²⁺ responses of rat bipolar cell terminals. Calcium imaging methods have previously been used to study Ca²⁺ responses at giant goldfish bipolar cell terminals (Heidelberger et al., 1991, 1992; Tachibana et al., 1993). However, to our knowledge, this

is the first calcium imaging study carried out in mammalian retinal bipolar cell terminals. Moreover, we were able to characterize the dynamics of the Ca²⁺ response at bipolar terminals, which have been shown to directly correlate with the release of neurotransmitters (Tachibana et al., 1993; Gersdorff and Matthews, 1994).

Properties of calcium responses at bipolar cell terminals

Our studies show that high K⁺ evokes a sustained Ca²⁺ response at bipolar cell terminals. The increase in [Ca²⁺]_i was due at least in part to Ca²⁺ influx upon K⁺ depolarization (we cannot exclude the possibility of Ca²⁺-induced intracellular Ca²⁺ release). We also demonstrated that the influx of Ca²⁺ occurs mainly via dihydropyridine-sensitive (L-type) Ca²⁺ channels, since nimodipine, a specific L-type Ca²⁺ channel antagonist, eliminated most of the K⁺-evoked Ca²⁺ response. This result is consistent with the sustained nature of the Ca²⁺ response. It is interesting to note that in mouse bipolar cells only a transient (T-type) Ca²⁺ channel was observed, while in goldfish bipolar cells a sustained (L-type) Ca²⁺ channel accounted for most of the Ca²⁺ influx (Kaneko et al., 1989; Heidelberger and Matthews, 1992; Tachibana et al., 1993).

Pharmacology of the GABA_C receptor of rat bipolar cell terminals

Our results show that both GABA_A and GABA_C receptors are involved in regulating the Ca²⁺ response of bipolar cell terminals. The pharmacologic profile of the GABA_C receptor of bipolar cell terminals to TACA and CACA is consistent with the findings of Feigenspan et al. (1993) using patch-clamp recording. This profile is similar to the so-called GABA_C receptor in fish and amphibian retinal cells (Qian and Dowling, 1993a, 1994; Dong et al., 1994; Lukasiewicz et al., 1994; Matthews et al., 1994; Zhang and Slaughter, 1994).

Figure 6. 3-APMPA does not antagonize the inhibitory effects of THIP or P4S. *A* and *B*, One hundred micromolar THIP or 100 μ M P4S suppressed K^+ -evoked Ca^{2+} responses. *C* and *D*, One hundred micromolar bicuculline completely blocked the inhibitory effects of THIP (100 μ M) and P4S (100 μ M). *E* and *F*, Five hundred micromolar 3-APMPA did not antagonize the inhibition produced by THIP (100 μ M) or P4S (100 μ M).

In this study, we determined that 3-APMPA and 3-APA, two phosphate analogs of GABA, selectively antagonized the $GABA_C$ receptor but not the $GABA_A$ receptor. Our results are consistent with studies performed on the bicuculline/baclofen-insensitive GABA receptor encoded by bovine retinal mRNA injected into *Xenopus* oocytes (Woodward et al., 1993). More recently, the antagonist nature of 3-APA at the $GABA_C$ receptor also has been reported in fish horizontal cells (Qian and Dowling, 1994). The selective antagonist properties of 3-APMPA and 3-APA at the $GABA_C$ receptor may be useful in the study of the functional role of $GABA_C$ receptor in retina.

One unique property of the pharmacology of the rat bipolar cell $GABA_C$ receptor is its picrotoxin insensitivity. The $GABA_C$ receptor in our preparation is extremely resistant to picrotoxin. This clearly differentiates this $GABA_C$ receptor from that reported in retinal horizontal and bipolar cells of fish and amphibians (Qian and Dowling, 1993a, 1994; Dong et al., 1994; Lukasiewicz et al., 1994; Matthews et al., 1994). Interestingly, the

$GABA_C$ -like receptor expressed by GABAp subunits, which is sensitive to picrotoxin, was actually cloned from the human retina ($IC_{50} = 0.4 \mu$ M for 1 μ M GABA) (Cutting et al., 1991, 1992; Shimada et al., 1992). The $GABA_C$ -like receptor expressed in *Xenopus* oocytes with mRNA extracted from bovine retina is also sensitive to picrotoxin ($IC_{50} = 0.5 \mu$ M for 1 μ M GABA) (Polenzani et al., 1991; Woodward et al., 1992). Therefore, it is unlikely that the picrotoxin insensitivity is a unique property of the $GABA_C$ receptor in mammals. Whether this receptor is unique to retinal bipolar cells in mammals is uncertain because rat bipolar cells are the only mammalian retinal neurons that have been studied so far in this manner. Further studies will be required to understand this apparent discrepancy and the underlying molecular mechanism.

Bicuculline sensitivity has been used as a major criteria to distinguish $GABA_A$ versus $GABA_C$ receptors (Johnston, 1986; Shimada et al., 1992). With the similarity of the pharmacologic profile of the $GABA_C$ receptor described here in rat to that of

Figure 7. Effects of TACA, muscimol, and CACA on K⁺-evoked Ca²⁺ responses. *A*, One millimolar TACA, in the presence of 100 μ M bicuculline, produced an inhibitory effect on Ca²⁺ responses similar to GABA. *B*, two millimolar muscimol, in the presence of 100 μ M bicuculline, did not produce substantial inhibition of the Ca²⁺ response. *C*, Five millimolar muscimol, in the presence of 100 μ M bicuculline, produced a delayed inhibition of the Ca²⁺ response. *D*, One hundred millimolar CACA, in the presence of 100 μ M bicuculline, had a somewhat variable inhibitory effect on the Ca²⁺ response. *E*, Two hundred millimolar CACA, in the presence of 100 μ M bicuculline, completely suppressed the Ca²⁺ response. *F*, The effect of 200 μ M CACA was blocked by 200 μ M 3-APMPA. *G*, The effect of 500 μ M CACA was not blocked by 500 μ M 3-APMPA. *H*, The effect of 500 μ M CACA was antagonized by the combination of 500 μ M 3-APMPA and 100 μ M bicuculline.

GABA_C receptors described in other species, with the exception of its extreme picrotoxinin resistance, it may be appropriate to refer to this receptor as a picrotoxin-insensitive GABA_C receptor. Alternatively, a new nomenclature, such as GABA_D, could be adopted, but we discourage this until the underlying molecular composition of this family of GABA receptor subunits is known.

Coexisting multiple receptors at bipolar cell terminals

Our results demonstrate that at least two types of GABA receptors, a GABA_A and a picrotoxin-insensitive GABA_C receptor, coexist in rat bipolar cell terminals, consistent with the findings of Feigenspan et al. (1993) on bipolar cell somas. Coexistence of GABA_A and GABA_C receptors at bipolar cell terminals has also been reported in tiger salamander and white perch (Qian and Dowling, 1993b; Lukasiewicz et al., 1994). Moreover, a novel baclofen-insensitive GABA_B-like receptor, found in gold-

fish bipolar cell terminals, was reported to be sensitive to sub-micromolar CACA and could directly modulate Ca²⁺ channels (Heidelberger and Matthews, 1991; Matthews et al., 1994). However, our calcium imaging methods did not reveal this novel GABA_B-like receptor in our preparation, since CACA was ineffective at low concentrations. We also failed to detect a baclofen-sensitive GABA_B receptor in our preparation, although such a receptor has also been reported to modulate Ca²⁺ channels at tiger salamander bipolar cell terminals (Maguire et al., 1989) and goldfish ganglion cells (Bindokas and Ishida, 1991), and to regulate retinal signal processing (Pan and Slaughter, 1991; Slaughter and Pan, 1992). It is not clear if this discrepancy is due to species variation or methodological differences.

Distinct properties of GABA_A and GABA_C receptors

In this study, we directly demonstrate that both GABA_A and the GABA_C receptors are involved in regulating the voltage-depen-

Figure 8. Comparison of effects of GABA on GABA_A receptors and GABA_C receptors. Inhibitory effects on K⁺-evoked Ca²⁺ responses produced by GABA acting at GABA_C receptors were isolated by coapplying 100 μM bicuculline and 40 μM picrotoxinin (A, C, E, G). Effects produced by GABA acting at GABA_A receptors were isolated by coapplying 200 μM 3-APMPA (B, D, F, H). Each GABA concentration was tested under both sets of conditions on the same bipolar cell terminal.

dent Ca²⁺ response at bipolar cell terminals. However, these two receptors display distinct affinities and activation kinetics. The GABA_C receptor has a high affinity for GABA. The inhibitory effect produced by this receptor was observed at submicromolar concentrations. In contrast, activation of GABA_A receptors required relatively high concentrations of GABA. The high potency of GABA at the GABA_C receptor is consistent with previous electrophysiological studies (Polenzani et al., 1991; Kusama et al., 1993a; Qian and Dowling, 1993a; Matthews et al., 1994).

While the GABA_C receptor has a much higher affinity, it clearly displays a delayed onset and slow time course of Ca²⁺ inhibition. This delay is still prominent at GABA concentrations approximately 50–100-fold greater than the threshold concentration. This is in contrast to the GABA_A receptor, which mediates

suppression of the early portion of the Ca²⁺ response at 5 μM GABA (Fig. 8D). This concentration is not much greater than the 1–2 μM needed to observe a barely detectable inhibitory effect, consisting of a reduction in the rate of rise of the initial Ca²⁺ response (Figs. 5B, 8B).

Fast activation kinetics is a property of most ionotropic ligand-gated ion channels. Although the GABA_C receptor has been reported to gate a chloride conductance (Feigenspan et al., 1993; Qian and Dowling, 1993a), slow activation of this receptor has not been previously described. The physiological implications of the slow onset of activity of the picrotoxin-insensitive GABA_C receptor described here are not yet clear. Further studies are needed to explain its phenomenon and its underlying mechanism.

Functional implications of the coexistence of multiple GABA receptors in bipolar cell terminals

It has been postulated that negative feedback from GABAergic amacrine cells to bipolar cell terminals is the major mechanism generating transient responses in the inner retina. What, then, is the role of the GABA_C receptor, and the implications of colocalization of GABA_A and GABA_C receptors at bipolar cell terminals? Based on the distinct properties of GABA acting at these two GABA receptor subtypes, one apparent explanation for the colocalization of multiple GABA receptors is fine tuning of the Ca²⁺ level in the bipolar cell terminals. The fast activation of the GABA_A receptor could generate fast, transient inhibition. However, this effect would lack a wide dynamic range due to its narrow activation parameters. On the other hand, the GABA_C receptor, with its slow activation kinetics and broad activation range, could well generate slow, sustained, and graded responses. Of course, this is based on the assumption that the synaptic concentration of GABA is regulated at micromolar levels. The concentration range of GABA at bipolar synaptic terminals is not clear. Lukasiewicz and Werblin (1994) have reported that light-evoked inhibitory synaptic inputs to bipolar cells in tiger salamander are not significantly antagonized by bicuculline. This result may imply that the light-evoked GABA concentration at bipolar cell terminals is in the low micromolar range and, therefore, not high enough to activate GABA_A receptors. An alternative interpretation for their results is also quite possible, as proposed by these authors (Lukasiewicz and Werblin, 1994). They postulated that transmission from bipolar cells to ganglion cells is primarily modulated by GABA_C receptors in the tiger salamander retina. Further studies, especially using selective GABA_C antagonists, will be necessary to answer this question.

The bipolar cells we studied are probably rod bipolar cells based on previous reports (Karschin and Wässle, 1990). Therefore, it is not clear whether there is a similar colocalization of GABA_A and GABA_C receptors at terminals other than rod bipolar cells. In tiger salamander retinal slice, it has been reported that there is no significant difference in GABA receptor pharmacology between ON and OFF bipolar cells (Lukasiewicz et al., 1994). However, in the same preparation, Zhang and Slaughter (1994) recently reported that activation of GABA_C receptors preferably blocked the ON response in third order retinal neurons, raising the possibility of a differential role of the GABA_C receptor in the ON and OFF pathways in the retina. Further studies will be necessary to define further the physiological influence of the GABA_C receptor at the retinal bipolar–ganglion cell synapse.

References

- Bindokas VP, Ishida AT (1991) (–)-Baclofen and γ -aminobutyric acid inhibit calcium currents in isolated retinal ganglion cells. *Proc Natl Acad Sci USA* 88:10759–10763.
- Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR, Kazian HH Jr (1991) Cloning of the γ -aminobutyric acid (GABA) ρ 1 cDNA: a GABA receptor subunit highly expressed in the retina. *Proc Natl Acad Sci USA* 88:2673–2677.
- Cutting GR, Curristin S, Zoghbi H, O'Hara B, Seldin ME, Uhl GR (1992) Identification of a putative γ -aminobutyric acid (GABA) receptor subunit ρ 2 and colocalization of the genes encoding ρ 2 (GABRR2) and ρ 1 (GABRR1) to human chromosome 6q14–q21 and mouse chromosome 4. *Genomics* 12:801–806.
- Dong C-J, Picaud SA, Werblin FS (1994) GABA transporters and GABA_C-like receptors on catfish cone- but not rod-driven horizontal cells. *J Neurosci* 14:2648–2658.
- Feigenspan A, Wässle H, Bormann J (1993) Pharmacology of GABA receptor channels in rat retinal bipolar cells. *Nature* 361:159–162.
- Gersdorff H, Matthews G (1994) Dynamics of synaptic vesicle fusion and membrane retrieval in synaptic terminals. *Nature* 367:735–739.
- Heidelberger R, Matthews G (1991) Inhibition of calcium influx and calcium current by γ -aminobutyric acid in single synaptic terminals. *Proc Natl Acad Sci USA* 88:7135–7139.
- Heidelberger R, Matthews G (1992) Calcium influx and calcium current in single synaptic terminals of goldfish retinal bipolar neurons. *J Physiol (Lond)* 447:235–256.
- Johnston GAR (1986) Multiplicity of GABA receptors. In: *Receptor biochemistry and methodology*, Vol 5, Benzodiazepine/GABA receptors and chloride channels (Olsen RW, Venter JC, eds), pp 57–71. New York: Liss.
- Kaneko A, Pinto LH, Tachibana M (1989) Transient calcium current of retinal bipolar cells of the mouse. *J Physiol (Lond)* 410:613–629.
- Kao JPY, Harootyan AT, Tsien RY (1989) Photochemically generated cytosolic calcium pulses and their detection by fluo-3. *J Biol Chem* 264:8179–8184.
- Karschin A, Wässle H (1990) Voltage- and transmitter-gated currents in isolated rod bipolar cells of rat retina. *J Neurophysiol* 63:860–876.
- Kusama T, Spivak CE, Whiting P, Dawson VL, Schaeffer JC, Uhl GR (1993a) Pharmacology of GABA ρ 1 and GABA_A receptors expressed in *Xenopus* oocytes and COS cells. *Br J Pharmacol* 109:200–206.
- Kusama T, Wang T-L, Guggino WB, Cutting GR, Uhl GR (1993b) GABA ρ 2 receptor pharmacological profile: GABA recognition site similarities to ρ 1. *Eur J Pharmacol* 245:83–84.
- Leifer D, Lipton SA, Barnstable CJ, Masland RH (1984) Monoclonal antibody to Thy-1 enhances regeneration of processes by rat retinal ganglion cells in culture. *Science* 224:303–306.
- Lipton SA, Tauck DL (1987) Voltage-dependent conductances of solitary ganglion cells dissociated from the rat retina. *J Physiol (Lond)* 385:361–391.
- Lukasiewicz PD, Werblin FS (1994) A novel GABA receptor modulates synaptic transmission from bipolar to ganglion and amacrine cells in the tiger salamander retina. *J Neurosci* 14:1213–1223.
- Lukasiewicz PD, Maple BR, Werblin FS (1994) A novel GABA receptor on bipolar cell terminals in the tiger salamander retina. *J Neurosci* 14:1202–1212.
- Maguire G, Maple B, Lukasiewicz P, Werblin F (1989) γ -aminobutyrate type B receptor modulation of L-type calcium channel current at bipolar cell terminals in the retina of the tiger salamander. *Proc Natl Acad Sci USA* 86:10144–10147.
- Massey SC, Redburn DA (1987) Transmitter circuits in the vertebrate retina. *Prog Neurobiol* 28:55–96.
- Matthews G, Ayoub GS, Heidelberger R (1994) Presynaptic inhibition by GABA is mediated via two distinct GABA receptors with novel pharmacology. *J Neurosci* 14:1079–1090.
- Nistri A, Sivilotti L (1985) An unusual effect of γ -aminobutyric acid on synaptic transmission of frog tectal neurones *in vitro*. *Br J Pharmacol* 85:917–921.
- Olsen RW, Venter JC (1986) Benzodiazepine/GABA receptors and chloride channels: structural and functional properties. In: *Receptor biochemistry and methodology*, Vol 5. New York: Liss.
- Pan Z-H, Lipton SA (1994a) Effect of NMDA receptor stimulation versus voltage-dependent calcium channel stimulation on calcium transient, and compartments in mammalian retinal ganglion cells. *Invest Ophthalmol Vis Sci [Suppl]* 35:1909.
- Pan Z-H, Lipton SA (1994b) Multiple GABA receptor subtypes mediate inhibition of calcium influx at rat retinal bipolar cell terminals. *Soc Neurosci Abstr* 20:217.
- Pan Z-H, Slaughter MM (1991) Control of retinal information coding by GABA_B receptor. *J Neurosci* 11:1810–1821.
- Polenzani L, Woodward RM, Miledi R (1991) Expression of mammalian γ -aminobutyric acid receptors with distinct pharmacology in *Xenopus* oocytes. *Proc Natl Acad Sci USA* 88:4318–4322.
- Qian H, Dowling JE (1993a) Novel GABA responses from rod-driven retinal horizontal cells. *Nature* 361:162–164.
- Qian H, Dowling JE (1993b) GABA responses on retinal bipolar cells. *Biol Bull* 185:312.
- Qian H, Dowling JE (1994) Pharmacology of novel GABA receptors

- found on rod horizontal cells of the white perch retina. *J Neurosci* 14:4299–4307.
- Shimada S, Cutting G, Uhl GR (1992) γ -aminobutyric acid A or C receptor? γ -aminobutyric acid $\rho 1$ receptor RNA induces bicuculline-, barbiturate-, and benzodiazepine-insensitive γ -aminobutyric acid responses in *Xenopus* oocytes. *Mol Pharmacol* 41:683–687.
- Sivilotti L, Nistri A (1989) Pharmacology of a novel effect of γ -aminobutyric acid on synaptic transmission of frog tectal neurones *in vitro*. *Eur J Pharmacol* 164:205–212.
- Sivilotti L, Nistri A (1991) GABA receptor mechanisms in the central nervous system. *Prog Neurobiol* 36:35–92.
- Slaughter MM, Pan Z-H (1992) The physiology of GABA_B receptors in the vertebrate retina. *Prog Brain Res* 9:47–60.
- Suzuki S, Tachibana M, Kaneko A (1990) Effects of glycine and GABA on isolated bipolar cells of the mouse retina. *J Physiol (Lond)* 421:645–662.
- Tachibana M, Kaneko A (1987) γ -Aminobutyric acid exerts a local inhibitory action on the axon terminal of bipolar cells: evidence for negative feedback from amacrine cells. *Proc Natl Acad Sci USA* 84:3501–3505.
- Tachibana M, Okada T, Arimura T, Kobayashi K, Piccolino M (1993) Dihydropyridine-sensitive calcium current mediates neurotransmitter release from bipolar cells of the goldfish retina. *J Neurosci* 13:2898–2909.
- Tauck DL, Frosch MP, Lipton SA (1988) Characterization of GABA- and glycine-induced currents of solitary rodent retinal ganglion cells in culture. *Neuroscience* 27:193–203.
- Woodward RM, Polenzani L, Miledi R (1992) Characterization of bicuculline/baclofen-insensitive γ -aminobutyric acid receptors expressed in *Xenopus* oocytes. I. Effects of Cl⁻ channel inhibitors. *Mol Pharmacol* 42:165–173.
- Woodward RM, Polenzani L, Miledi R (1993) Characterization of bicuculline/baclofen-insensitive (ρ -like) γ -aminobutyric acid receptors expressed in *Xenopus* oocytes. II. Pharmacology of γ -aminobutyric acid_A and γ -aminobutyric acid_B receptor agonists and antagonists. *Mol Pharmacol* 43:609–625.
- Yeh HH, Lee MB, Cheun JE (1990) Properties of GABA-activated whole-cell currents in bipolar cells of the rat retina. *Vis Neurosci* 4:349–357.
- Zhang J, Slaughter MM (1994) The GABA ρ receptor preferentially suppresses ON responses in the amphibian retina. *Invest Ophthalmol Vis Sci [Suppl]* 35:1364.