Subcellular Localization and Complements of GABA_A and GABA_C Receptors on Bullfrog Retinal Bipolar Cells

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Du, Jiu-Lin and Xiong-Li Yang. Subcellular localization and complements of GABA_A and GABA_C receptors on bullfrog retinal bipolar cells. J Neurophysiol 84: 666–676, 2000. γ-Aminobutyric acid (GABA) receptors on retinal bipolar cells (BCs) are highly relevant to spatial and temporal integration of visual signals in the outer and inner retina. In the present work, subcellular localization and complements of GABA_A and GABA_C receptors on BCs were investigated by whole cell recordings and local drug application via multi-barreled puff pipettes in the bullfrog retinal slice preparation. Four types of the BCs (types 1–4) were identified morphologically by injection of Lucifer yellow. According to the ramification levels of the axon terminals and the responses of these cells to glutamate (or kainate) applied at their dendrites, types 1 and 2 of BCs were supposed to be OFF type, whereas types 3 and 4 of BCs might be ON type. Bicuculline (BIC), a GABA_A receptor antagonist, and imidazole-4-acetic acid (I4AA), a GABA_C receptor antagonist, were used to distinguish GABA receptor-mediated responses. In all BCs tested, not only the axon terminals but also the dendrites showed high GABA sensitivity mediated by both GABA_A and GABA_C receptors. Subcellular localization and complements of GABA_A and GABA_C receptors at the dendrites and axon terminals were highly related to the dichotomy of OFF and ON BCs. In the case of OFF BCs, GABA_A receptors were rather evenly distributed at the dendrites and axon terminals, but GABA_C receptors were predominantly expressed at the axon terminals. Moreover, the relative contribution of GABA_A receptors to the axon terminals was prevalent over that of GABA_C receptors, while the situation was reversed at the dendrites. In the case of ON BCs, GABA_A and GABA_C receptors both preferred to be expressed at the axon terminals; relative contributions of these two GABA receptor subtypes to both the sites were comparable, while GABA_C receptors were much less expressed than GABA_A receptors. GABA_A, but not GABA_C receptors, were expressed clusteringly at axons of a population of BCs. In a minority of BCs, I4AA suppressed the GABA_C responses at the dendrites, but not at the axon terminal, implying that the GABA_C receptors at these two sites may be heterogeneous. Taken together, these results suggest that GABA_A and GABA_C receptors may play different roles in the outer and inner retina and the differential complements of the two receptors on OFF and ON BCs may be closely related to physiological functions of these cells.

INTRODUCTION

Recent experimental and model studies have clearly demonstrated that for a specific kind of ion channels, not only their characteristics but also their subcellular spatial distribution are of utmost relevance to physiological functions of the cells (for review, see Magee et al. 1998; Nusser and Somogyi 1997; Safronov 1999). Bipolar cells (BCs) are second-order neurons, which are involved in information processing in the outer and inner plexiform layers (OPL and IPL) of the vertebrate retina (Dowling 1987). γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter that modulates synaptic transmission at both synaptic layers (Barnstable 1993; Lukasiewicz and Shields 1998a; Wu 1992; Yazulla 1986). It is generally thought that BCs receive input from GABAergic horizontal cells in the OPL, which may mediate the surround response of the receptive field of BCs via feedback inhibition onto photoreceptors (Wu 1992) and/or feedforward inhibition onto BC dendrites (Dowling and Werblin 1969; Hare and Owen 1992; Lasansky 1973, 1980; Yang and Wu 1991). Furthermore GABAergic amacrine cells are supposed to feedback to BC axon terminals through the reciprocal synapses, which may be responsible for temporal modulation of BC output signals (Dong and Werblin 1998; Du and Yang 1999a; Hartveit 1999). It is evident, therefore that spatial distribution of GABA receptors on BCs is crucial for spatial and temporal integration of visual signals in the outer and inner retina. The results concerning subcellular localization of GABA receptors on BCs seem to be inconsistent. While GABA receptor have been demonstrated morphologically (Enz et al. 1995, 1996; Fletcher et al. 1998; Koulen et al. 1997, 1998; for review, see Wässle et al. 1998) and physiologically (Euler and Wässle 1998; Feigenspan et al. 1993; Karschin and Wässle 1990; Lukasiewicz and Wong 1997; Lukasiewicz et al. 1994; Maple and Wu 1996; Tachibana and Kaneko 1987) to be localized exclusively on BC axon terminals in most vertebrate retinas, GABA receptor-mediated currents were reported to be induced from both dendrites and axon terminals of BCs in several species (Qian and Dowling 1995; Suzuki et al. 1990). Our first goal was to re-examine this issue, using a bullfrog retinal slice preparation.

In addition to GABA_A and GABA_B receptors, a novel GABA_C receptor has recently been identified (Feigenspan et al. 1993; Qian and Dowling 1993). GABA_A and GABA_C receptors are both ionotropic receptors incorporating chloride channels, but they have distinct kinetics and may mediate signal transfer in different time domains in the retina (Han and Yang 1999; Lukasiewicz and Shields 1998a). A second goal of the

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present work was to study complements of GABA<sub>A</sub> and GABA<sub>C</sub> receptors on BCs to gain an insight into the physiological roles of these receptor subtypes for information processing of BCs.

In both mammalian and nonmammalian retinas, distinct subtypes of BCs have been identified morphologically and physiologically. Several lines of evidence indicate that orn-type BCs, which are hyperpolarized by illumination of the receptive field center, have telodendria ramifying in the distal IPL (sublamina a), while on-type BCs, which are depolarized by illumination of their receptive field centers, have telodendria ramifying in the proximal IPL (sublamina b) (Dowling 1987; Euler and Wässle 1995; Hare et al. 1986). Furthermore these two subtypes show different responses to glutamate, mediated by distinct subtypes of glutamate receptors (for review, see Massey and Maguire 1995; Shiells and Falk 1995; Wilson 1994). A final purpose of this work was to determine how relative contributions of GABA<sub>A</sub> and GABA<sub>C</sub> receptors were related to the dichotomy of off and on BCs in the bullfrog retina. Part of this study has appeared in abstract form (Du and Yang 1998).

**METHODOLOGY**

**Retinal slice preparation**

Adult bullfrogs (*Rana catesbeiana*), maintained in tanks at 4°C on a 12-h light/12-h dark cycle, were used in the present work. Retinal slices were prepared following the procedures reported previously (Werblin 1978; Wu 1987) with minor modifications (Du and Yang 1999b). In brief, prior to an experiment an animal was dark-adapted for at least 1 h. After the animal was pithed and decapitated, an eye was enucleated, and the cornea and lens were removed. The eyecup thus formed was immersed with the standard bullfrog Ringer solution (see *Solutions and drug application* for the composition) and quanti-

**Optical setup and recording chamber**

The recording chamber was placed on a fixed stage microscope (Zeiss, ACM, Germany), which was equipped with epifluorescence illumination and Hoffman modulation contrast optics (Hoffman Modulation Optics, Greenvale, NY). A water-immersion objective with a working distance of 2.9 mm was used (Zeiss, X40, 0.75 NA), with its metal parts being coated with Epon to avoid corrosion and contact voltages. During the experiments, the cells were imaged by an infrared video camera and visualized on a TV monitor. Additional magnification was obtained by a ×3 lens (Zeiss Optovar) inserted in the imaging pathway.

The recording chamber had a volume of ~0.8 ml and was continuously perfused with the oxygen-bubbled extracellular solution. The solution was fed in and out of the recording chamber with a peristaltic pump (Minipulse 3, Gilson Medical Electronics, Villiers-le-Bel, France) at a rate of 2–3 ml/min.

**Whole cell recordings**

Recording patch electrodes were pulled from borosilicate glass (Shanghai Brain Research Institute, Shanghai, China) with a two-stage vertical puller (PB-7, Narishige, Japan). The resistance of the electrodes was 8–12 MΩ in the bathing medium when filled with the intracellular solution whose composition is given in the following text. Connected to the amplifier (CEZ-2300, Narishige, Japan) via an Ag/AgCl wire, the recording electrode was mounted on a mechanical micromanipulator (MMN-9, Narishige, Japan). To get a large angle between the recording electrode and the slice, the pipette was visually bent for ~30–50° at 0.5–0.8 mm away from the tip by a heating platinum wire under microscopy before the experiments. Fine adjustment of the electrode was made possible with the aid of a hydraulic micromanipulator (MHW-3, Narishige, Japan). The reference electrode was an Ag/AgCl wire connected to the recording chamber. The liquid junction potential of the recording electrode was measured (Neher 1992) and routinely corrected.

Cells on the surface of the slices could be easily visualized with the aid of the optical setup. The action of the fluid stream coming out of the electrode tip driven by a positive pressure applied to the electrode was sufficient for seal formation, and no extra cleaning of cell surface was needed. In most cases, whole cell recordings (Hamill et al. 1981) were only made from cells located at, or a little below, the surface of the slices. Gigaohm (2–10 GΩ) sealing between the electrode tip and the cell membrane was obtained by positioning the electrode tip onto the cell surface, releasing the positive pressure, and applying careful suction. The whole cell configuration was established by rupturing the membrane with brief pulses of suction and/or in combination with brief voltage transients applied to the electrode. The series resistance of the recording electrode estimated from the peak amplitude of the capacitive current was reduced to ~10 MΩ with resistance compensation, which would produce a holding voltage error of <2 mV when the currents recorded were typically ~200 pA. Capacitative currents caused by the electrode and cell capacitance were partially cancelled by the circuit of the amplifier. pClamp 6.0.4 (Axon instruments, Foster City, CA) was used to generate voltage command outputs, acquire data, and trigger drug application puff via a DigiData 1200A (Axon instruments) interface on an IBM-compatible personal computer. The data were low-pass filtered with a fourth Bessel filter at 1 kHz and digitized at 2.5 kHz. Data statistical analysis was performed using Student’s paired test.

To classify BCs morphologically, the patch electrodes were filled with Lucifer yellow (0.1%), which could diffuse into the BCs during the recordings when the whole cell configuration was established so that both cell bodies and fine processes were clearly visible by epifluorescent illumination under microscopy. The experiments were performed under room illumination and the slices were very likely light adapted.

**Solutions and drug application**

The standard intracellular solution with an osmolality of 290 mOsm consisted of (in mM) 100.5 cesium fluoride, 40 cesium chloride, 3 sodium chloride, 0.4 magnesium chloride, 0.1 calcium chloride, 10 HEPES, 1 EGTA, 3 Mg<sub>2</sub>ATP, and 0.5 Na<sub>3</sub>GTP, adjusted to pH 7.7 with cesium hydroxide. The standard bath medium, which was only used for the preparation of retinal slices, contained (in mM) 150 sodium chloride, 2 potassium chloride, 2 calcium chloride, 2 magnesium chloride, 10 HEPES, and 10 glucose, adjusted to pH 7.8 with sodium hydroxide, with an osmolality of ~310 mOsm. Since cobalt ions can substantially suppress the responses mediated by GABA receptors (Kaneda et al. 1997; Kaneko and Tachibana 1986), we instead used 20 mM magnesium chloride, substituting for an osmotically equivalent amount of sodium chloride and 2 mM calcium chloride to block synaptic transmission (Łukasiwicz and Werblin 1994; Łukasiwicz et al. 1994) in all the experiments reported in this
paper. In the high Mg solution, neither spontaneous excitatory/inhibitory postsynaptic current (EPSC/IPSC) nor calcium currents could be recorded from BCs, proving that these cells received no synaptic inputs. Glutamate (GLU), kainate (KA), 6-cyanoquinoxaline-2,3-dione (CNQX), γ-aminobutyric acid (GABA), picrotoxin (PTX), bicuculline (BIC), and imidazole-4-acetic acid (I4AA) were obtained from Research Biochemicals (RBl, MA). All other chemicals were purchased from Sigma Chemicals (St. Louis).

When subcellular localization of GABA receptors was studied, drugs were locally applied to a specific subcellular site of the recorded BC using seven-barrelled pneumatic puff pipettes with a hydraulic micromanipulator (MO-330, Narishige, Japan). The puff pipettes were pulled from borosilicate glass (CG-17, Shanghai Brain Research Institute, Shanghai, China) with a multi-stage vertical puller (Shanghai Institute of Physiology, Shanghai, China), and the tip of each pipette was 3–5 μm in diameter. These pipettes were positioned in parallel with the longitude axis of the retinal slice and in a direction opposite to the bath medium flow in the chamber. Drugs were pressure ejected by nitrogen gas with a pressure of 4–8 psi via a Picospritzer II (General Valve, Fairfield, NJ), which was triggered by the pClamp software. In control experiments, all seven barrels were filled with the same solution to make sure that each barrel was positioned correctly to optimize consistency of drug delivery between barrels, and differences of <10% in whole cell currents were found with drug delivery given by different barrels. When no pressure was applied to the pipettes, small amount of the bath medium was continuously sucked into the pipettes by capillary attraction, which prevented the test solutions from leaking out. The concentrations of drugs given throughout the text refer to the concentrations in barrels of the puff pipettes and were chosen in reference to the results of preliminary experiments. They were 1 mM for GLU, 0.2 mM for KA, 0.05 mM for CNQX, 1 mM for GABA, 0.5 mM for PTX, 0.5 mM for BIC, and 2 mM for I4AA. The actual concentrations at the cell membrane were surely much lower because of bulk flow, diffusion, and potent uptake systems in the retina.

RESULTS

Morphological and functional identification and classification of bullfrog BCs

With intracellular Lucifer yellow staining, four types of BCs could be distinguished according to the ramification levels of their axons within the IPL and, for brevity, they are referred to as types 1–4 of BCs in the present work. The classification scheme is shown in Fig. 1, and the cells are numbered according to the branching patterns and the ramification levels of their axon terminals within the inner plexiform layer (IPL) and are arrayed accordingly. Types 1 and 2, stratifying in sublamina a, may be OFF BCs, whereas types 3 and 4, stratifying in sublamina b, may be ON BCs, —, the border between sublamina a and b. Both sublamina a and b were subdivided into 2 strata of equal widths, - - - , the borders of 2 neighboring strata. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.

FIG. 1. Classification scheme for bullfrog retinal bipolar cells (BCs). Camera lucida drawings from Lucifer yellow staining cells show the BC types present in the bullfrog retina. Four different types of BCs (labeled 1–4) are classified according to the branching patterns and the ramification levels of their axon terminals with the inner plexiform layer (IPL) and are arrayed accordingly. Types 1 and 2, stratifying in sublamina a, may be OFF BCs, whereas types 3 and 4, stratifying in sublamina b, may be ON BCs, —, the border between sublamina a and b. Both sublamina a and b were subdivided into 2 strata of equal widths, - - - , the borders of 2 neighboring strata. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.

The preceding morphological classification of OFF and ON BCs was further confirmed by examining the responses induced by the application of glutamate to BC dendrites. It is generally thought that at their dendrites, OFF BCs express ionotropic glutamate receptors (iGluRs), whereas ON BCs express metabotropic glutamate receptors (mGluRs) in a variety of vertebrate retinas (Euler et al. 1996; for review, see Massey and Maguire 1995; Shiells and Falk 1995; Wilson 1994).

When glutamate receptor agonists were locally applied to the dendrites of BCs, two types of responses were observed that were closely related to the stratification levels of the axon terminals of these cells: sublamina a or b. Whole cell recordings from a type 1 BC and a type 3 BC identified morphologically are shown in Fig. 2. Dendritic puff application of kainate (200 μM), an agonist of iGluRs, to the type 1 cell (labeled I in Fig. 2A) evoked an inward sustained current with a reversal potential of about +1 mV, which could be completely blocked in the presence of CNQX (50 μM), a non-N-methyl-D-aspartate (NMDA) receptor antagonist (Fig. 2B). Similar results were obtained with glutamate application, except that the glutamate-induced currents were more transient (data not shown). Such currents were recorded from all types 1 and 2 of BCs tested (18/18) without exception, which further strengthened the suggestion that types 1 and 2 were OFF BCs. For the type 3 BC (labeled II in Fig. 2A), glutamate (1 mM) evoked an outward current (Fig. 2C) with a negative slope resistance and a reversal potential of about −5 mV. Following co-application of CNQX, the current only slightly decreased in size, suggesting that the current was mainly mediated by mGluRs. Similar results were
observed in 5 of 11 type 3 and 4 BCs examined. In the remaining six cells, glutamate elicited no currents at all; this may be due to the rundown of mGluR responses of ON BCs during the whole cell recordings, as often reported in previous work (Lasansky 1992; Lukasiewicz and Werblin 1994).

Spatial profile of GABA sensitivity of BCs

Spatial distribution of GABA receptors on bullfrog BCs was further explored by applying GABA locally at different subcellular sites via single-barreled puff pipettes. Drugs were locally applied at the BC dendrites via seven-barreled puff pipettes. B: characteristics of kainate (KA)-induced current from the dendrites of the type 1 BC (labeled I in A). Puff application of 0.2 mM KA evoked an inward sustained current with a reversal potential of +1 mV, as indicated by the I-V curve shown below the traces. The KA-induced current could be completely suppressed by co-application of 0.05 mM 6-cyanoquinoxaline-2,3-dione (CNQX). C: characteristics of glutamate (Glu)-induced current from the type 3 BC (labeled II in A). Puff application of 1 mM Glu at the dendrites evoked a CNQX-resistant outward current with a reversal potential of −5 mV, as indicated by the I-V curve shown below the traces. Both the cells were voltage-clamped at −60 mV, and the reversal potentials of the currents were determined by voltage ramps from −100 to +60 mV (500 mV/s). The puff duration (u) was 400 ms.

In the remaining six cells, glutamate elicited no currents at all; this may be due to the rundown of mGluR responses of ON BCs during the whole cell recordings, as often reported in previous work (Lasansky 1992; Lukasiewicz and Werblin 1994).

Spatial profile of GABA sensitivity of BCs

Spatial distribution of GABA receptors on bullfrog BCs was further explored by applying GABA locally at different subcellular sites via single-barreled puff pipettes with a fine tip (<1 μm ID; Fig. 3A). As shown in Fig. 3B, currents could be elicited from this type 3 BC when 1 mM GABA was puffed either at the dendrites (D), soma (S), axon (A), or axon terminal (T). The GABA-induced currents were relatively large at the dendrites (36 pA) and axon terminal (27 pA) but less at the axon (18 pA). The current was even detectable at the soma (6 pA). It may be argued that the current elicited by puffing GABA at the soma was simply a consequence of the activation of the GABA receptors at the dendrites and axon terminal by GABA diffusion. To clarify this issue, the soma and processes of the cell were gently elevated with the patch electrode (position II in Fig. 3A), but without destroying the whole cell recording configuration, to avoid possible diffusion effects as much as possible. Under such a condition, the response to local GABA application at the soma disappeared (Fig. 3C), while the other three responses to local GABA application to D, A, and T remained almost unchanged. This result implies that no or few GABA receptors are expressed on the soma. Similar experiment conducted in eight other BCs (3 OFF, 5 ON) yielded comparable results. In agreement with this suggestion, it was found that GABA failed to elicit any observable currents from outside-out membrane patches of the BC soma (n = 10, data not shown). All the responses to application of 1 mM GABA

FIG. 2. Identification of glutamate receptor subtypes on BC dendrites. A: a sketch showing 2 BCs in the retinal slice and the drug application system. Drugs were locally applied at the BC dendrites via seven-barreled puff pipettes. B: characteristics of kainate (KA)-induced current from the dendrites of the type 1 BC (labeled I in A). Puff application of 0.2 mM KA evoked an inward sustained current with a reversal potential of +1 mV, as indicated by the I-V curve shown below the traces. The KA-induced current could be completely suppressed by co-application of 0.05 mM 6-cyanoquinoxaline-2,3-dione (CNQX). C: characteristics of glutamate (Glu)-induced current from the type 3 BC (labeled II in A). Puff application of 1 mM Glu at the dendrites evoked a CNQX-resistant outward current with a reversal potential of −5 mV, as indicated by the I-V curve shown below the traces. Both the cells were voltage-clamped at −60 mV, and the reversal potentials of the currents were determined by voltage ramps from −100 to +60 mV (500 mV/s). The puff duration (u) was 400 ms.

FIG. 3. Spatial profile of GABA sensitivity on an ON BC. A: a sketch showing a type 3 BC in the retinal slice and the drug application system. GABA was applied at the dendrites (represented by D), soma (S), axon (A), and axon terminal (T) using single-barreled puff pipettes. Whole cell recordings were first made when the cell was located in situ (labeled I), and then the soma was raised and GABA was applied at the 4 sites again (labeled II). B and C: responses of the cell to puffs of 1 mM GABA at the 4 locations under the in situ condition (B) and when the soma was raised (C), respectively. D: when 0.5 mM picrotoxin (PTX) was added in the perfusing medium, all the responses to application of 1 mM GABA to the dendrites, axon, and axon terminal were completely blocked. The cell was held at −60 mV, and the puff duration (u) was 100 ms.
to the dendrites, axon, and axon terminal were completely blocked by 0.5 mM PTX (Fig. 3D), a classical blocker of chloride channels, in all BCs tested.

**Complements of GABA\(_A\) and GABA\(_C\) receptors at BC dendrites and axon terminals**

Since GABA\(_A\) and GABA\(_C\) receptors both are chloride channels, our next questions were to determine relative contributions of the currents mediated by GABA\(_A\) and GABA\(_C\) receptors to the total responses to GABA application at BC dendrites and axon terminals and how the current fractions were related to different BC types. For these purposes, BIC, a competitive GABA\(_A\)-receptor antagonist, and I4AA, a presumably competitive antagonist of GABA\(_C\) receptors (Han et al. 1997; Kusama et al. 1993; Picaud et al. 1998; Qian and Dowling 1994, 1995), were used to suppress the GABA\(_A\) and GABA\(_C\) response components, respectively, so that the fractions of these components could be determined. For a more quantitative estimate of these current fractions, two cautions should be considered. First, in consideration of the different affinities of GABA\(_A\) and GABA\(_C\) receptors for GABA (for review, see Feigenspan and Bormann 1998; Lukasiewicz and Shields 1998a), a saturating concentration of GABA should be used so that all GABA receptors are fully activated. It was found that the GABA-induced currents were no longer increased in amplitude with the increase of GABA concentration when it exceeded 0.5 mM in the puff pipette. Second, we must be sure that BIC or I4AA, co-applied with GABA, could fully suppress the GABA\(_A\) or GABA\(_C\) components of GABA-induced responses. We have found that suppression of the GABA\(_A\) or GABA\(_C\) components was not increased with a further increase of BIC or I4AA concentration when it was 0.5 mM for BIC and 2 mM for I4AA. Actually, as shown in Fig. 4, at the axon terminal of a type 2 BC, 1 mM GABA induced-current (bottom, —) is precisely the algebraic sum (bottom, - - -) of the currents respectively induced by co-application of 1 mM GABA with 2 mM I4AA (GABA\(_A\) component, top) and with 0.5 mM BIC (GABA\(_C\) component, middle). The ratio of the algebraic sum to the total GABA current was 0.92 ± 0.12 (mean ± SD; \(n = 8\), \(P > 0.05\)). In consequence, 1 mM GABA, 2 mM I4AA, and 0.5 mM BIC (all in the puff pipettes) were used as “standard” concentrations for this kind of analysis.

In most of BCs tested (87/90), GABA\(_A\) and GABA\(_C\) currents could be elicited from both dendrites and axon terminals. An example recorded from a type 1 BC is shown in Fig. 5. Two seven-barreled puff pipettes were, respectively, directed toward the dendrites and axon terminal of the cell, which was voltage-clamped at −60 mV. Co-application of GABA and I4AA at the dendrites and axon terminal elicited comparable inward currents (GABA\(_A\) responses, 38 pA vs. 46 pA; Fig. 5A). When GABA was co-applied with BIC, the current (GABA\(_C\) response) elicited from the axon terminal (74 pA) was significantly larger than that from the dendrites (11 pA; Fig. 5B). It is noteworthy that the GABA\(_C\) responses elicited from both the dendrites and axon terminal were more sustained than the GABA\(_A\) responses; this is in agreement with previous reports (Han et al. 1997; Qian and Dowling 1993, 1995; for review, see Feigenspan and Bormann 1998; Lukasiewicz and Shields 1998a). This may be due to the slower desensitization and deactivation of GABA\(_C\) receptors than the GABA\(_A\) receptors. The GABA responses at the two sites were almost completely blocked by co-application of I4AA and BIC (Fig. 5C). These results clearly demonstrate that GABA\(_A\) and GABA\(_C\) receptors are expressed at both the dendrites and axon terminals of the bullfrog BCs.

We have made this kind of analysis in 19 OFF (12 type 1 and 7 type 2), and 17 ON (7 type 3 and 10 type 4) BCs. Due to qualitative similarity, we did not made distinction between the data obtained from types 1 and 2 or types 3 and 4. All the data concerning the peak currents mediated by GABA\(_A\) and GABA\(_C\) receptors elicited from the dendrites and axon terminals of these cells are summarized in Table 1. It is evident that the relative contributions of the GABA\(_A\) and GABA\(_C\) receptors...
TABLE 1. Peak amplitudes of GABA_A and GABA_C receptor-mediated currents elicited at BC dendrites and axon terminals

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>n</th>
<th>GABA_A Currents, pA</th>
<th>GABA_C Currents, pA</th>
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<tbody>
<tr>
<td>Off bipolar cells</td>
<td>19</td>
<td>41 ± 22</td>
<td>39 ± 16</td>
</tr>
<tr>
<td>On bipolar cells</td>
<td>17</td>
<td>30 ± 12</td>
<td>62 ± 31</td>
</tr>
</tbody>
</table>

Data are represented as means ± SD.

to dendrites and axon terminals varied with the cell types: off or on types.

In Fig. 6A, the ratios of the currents elicited from the dendrites and axon terminals (D/A ratios) are represented by black (■) and white (□) bars, respectively for the GABA_A and GABA_C responses. In the case of off BCs, D/A ratio was 1.12 ± 0.53 (mean ± SD) for the GABA_A currents, indicating that the GABA_A currents elicited from the dendrites and axon terminals were almost equal (P > 0.05). The GABA_C currents of the dendrites were much smaller than those of the axon terminals (D/A = 0.14 ± 0.12, P < 0.001). In the case of on BCs, D/A ratio was 0.48 ± 0.23 for the GABA_A currents and 0.34 ± 0.21 for the GABA_C currents, showing that GABA_A and GABA_C receptors both prefer to be expressed at their axon terminals (P < 0.001).

At the dendrites or axon terminal of a single BC, the fractions of GABA_A and GABA_C receptor-mediated currents were found to be closely related to the cell types (Fig. 6B). For the off BCs, the relative GABA_C:GABA_A ratio was ~0.23 ± 0.07 at the dendrites (left ■), indicating that the dendrites of these cells may express GABA_C receptors much less than GABA_A receptors (P < 0.001). In contrast, as indicated by the high GABA_C:GABA_A ratio (2.46 ± 1.14) for the axon terminals (left □), GABA_C receptors may be much more expressed at this site (P < 0.001). For the on BCs, however, the GABA_C:GABA_A ratios were much less than 1.0 at both the dendrites (0.15 ± 0.05; right ■) and axon terminals (0.33 ± 0.09; right □). In other words, expression of GABA_A receptors at both dendrites and axon terminals prevailed over that of GABA_C receptors for the on BCs (P < 0.001).

GABA_A receptors at BC axons

As shown in Fig. 3, local application of GABA at the BC axon could evoke currents. One may argue that the currents induced from the axon were simply because GABA locally applied at the axon might have activated GABA receptors located at the axon terminal. To rule out this possibility, whole cell recordings were made from the BCs with soma and dendrites intact but axon terminals lost. For identification of the cell type that lost axon terminals, we also examined the properties of their responses to glutamate. In all these BCs (n = 9), GABA_A and GABA_C currents were elicited at their dendrites (Fig. 7, A and B, top), which were similar to the results obtained from the BCs with axon terminals intact (compare with Fig. 5). In four of nine cells (2 off and 2 on BCs), neither GABA_A nor GABA_C currents were elicited at their axons (Fig. 7A, bottom). However, in the remaining five cells (1 off and 4 on BCs), GABA_A responses were clearly evoked, while no GABA_C currents were detectable (Fig. 7B, bottom). These results demonstrate that GABA_A, but not GABA_C receptors, exist at the axons of a population of bullfrog BCs.

GABA_A receptors were not evenly distributed along BC axons. In the BCs (n = 3) with axon terminals lost, 1 mM GABA with 2 mM I4AA was applied, using fine single-barreled pipettes, onto four different locations, separated by equal distance (~15 μm), along the axons (Fig. 8A), and the currents were recorded and compared. In the example shown in Fig. 8B, the current induced at location A1 was relatively large, with a short delay and a fast rising phase. As the location was moved away from A1 and closer to the GCL, the induced currents became smaller, with the delay longer and the rising phase slower. The results obtained from the two other BCs were qualitatively similar, though the location at which the largest and fastest current was elicited was not necessarily the same. These results suggest that GABA_A receptors may be clustered on the BC axons. It was also noted from Fig. 8 that the current elicited from the soma was smaller than those elicited from A1 and A2. This observation eliminated the possibility that the currents induced by co-application of GABA and I4AA to the axon might be due to a result of the activation of GABA_A receptors on the dendrites by GABA diffusion.
Heterogeneity of GABA<sub>C</sub> receptors

In most cases (58/62), I4AA acted as an effective antagonist of GABA<sub>C</sub> receptors of bullfrog BCs. However, in four BCs (2 OFF and 2 ON), it was found that I4AA failed to effectively suppress the GABA<sub>C</sub> responses. Such an example is shown in Fig. 9. For this ON BC, addition of I4AA (2 mM) completely abolished the GABA current (GABA<sub>C</sub> response) remaining after co-application of BIC at the dendrites, but only slightly suppressed that at the axon terminal. This result raised a possibility that GABAC receptors at these two subcellular sites may have different pharmacological characteristics in a minority of bullfrog BCs.

DISCUSSION

In the present work, subcellular localization and complements of GABA<sub>A</sub> and GABA<sub>C</sub> receptors on morphologically and physiologically identified bullfrog retinal BCs were systematically examined. Our results showed that GABA receptors were expressed at both BC dendrites and axon terminals. We further examined subcellular localization and complements of GABA<sub>A</sub> and GABA<sub>C</sub> receptors at the dendrites and axon terminals. As seen in Figs. 4, 5, 7, and 9, the delay and the rate of rise of cell responses showed a relatively large variation. Such variation might be caused by the presence of intrinsic diffusion barriers of slice preparation, such as narrow extracellular spaces and uptake systems, and three-dimensional distribution of GABA receptors on BC processes, etc. Thus we used the peak amplitude of responses as an index for the estimation of relative contributions of these receptors. It was found that relative contributions of GABA<sub>A</sub> and GABA<sub>C</sub> receptors were clearly related to the dichotomy of OFF and ON BCs. In the case of OFF BCs, GABA<sub>A</sub> receptors were rather evenly distributed at the dendrites and axon terminals, but expression of GABA<sub>C</sub> receptors showed a bias in favor of the axon terminals; the relative contribution of GABA<sub>C</sub> receptors to the axon terminals was prevalent over that of GABA<sub>A</sub> receptors, while the situation was reversed for the dendrites. In the case of ON BCs, GABA<sub>A</sub> and GABA<sub>C</sub> receptors both preferred to be expressed at the axon terminals, and their relative contributions to either dendrites or axon terminals were comparable. Furthermore the relative contribution of GABA<sub>C</sub> receptors to both sites was much less than that of GABA<sub>A</sub> receptors. The results are summarized in the cartoon of Fig. 10.

FIG. 7. GABA<sub>A</sub> receptors at BC axons. A: currents elicited from an ON BC with the axon terminal lost. For this cell, currents could be evoked when 1 mM GABA was co-applied with 2 mM I4AA or 0.5 mM BIC at the dendrites (D), but not at the axon (A). B: currents elicited from another ON BC with the axon terminal lost. For this cell, currents could be elicited when 1 mM GABA was co-applied with either 2 mM I4AA or 0.5 mM BIC at the dendrites; At the axon currents could be only evoked when GABA was co-applied together with I4AA but not with BIC. The cells were held at −60 mV and the puff duration (ms) was 400 ms.

Heterogeneity of GABA<sub>C</sub> receptors

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Spatial distribution of GABA receptors on BCs

In all retinas studied immunocytochemically to date (Enz et al. 1995, 1996; Fletcher et al. 1998; Koulen et al. 1997, 1998; Lin and Yazulla 1994; for review, see Wässle et al. 1998), abundant evidence have shown that strong punctuate immunofluorescence of GABAA and GABA C receptor subunits was observed at the BC axon terminals but not in the OPL. It is thus suggested that GABAA and GABA C receptors are predominantly clustered at the axon terminals but not at the dendrites. In agreement with the anatomical results, a lot of electrophysiological work has demonstrated that GABA failed to induce currents from the BC dendrites, while GABA responses could be consistently elicited from the BC axon terminals (Euler and Wässle 1998; Feigenspan et al. 1993; Karschin and Wässle 1990; Lukasiewicz and Wong 1997; Lukasiewicz et al. 1994; Maple and Wu 1996; Tachibana and Kaneko 1987). On the other hand, there are also studies showing GABA-induced currents from BC dendrites in the retina of mouse (Suzuki et al. 1990) and hybrid bass (Qian and Dowling 1995). In the bullfrog retina, we have shown that GABA could induce large currents from the dendrites of most BCs tested that were comparable in amplitude with those induced from the axon terminals. This result strongly suggests that GABA receptors indeed exist on the BC dendrites. Since horizontal cells of many species (Yazulla 1986), including bullfrog (Zhang et al. 1999), are GABAergic and feedforward synapses exist between horizontal cells and BCs (Dowling and Werblin 1969; Hare and Owen 1992; Lasansky 1973, 1980; Yang and Wu 1991), it sounds reasonable to postulate that the dendritic GABA receptors may mediate feedforward synaptic inputs from horizontal cells to BCs and participate directly in the formation of the surround antagonism of the BC receptive fields.

It was of interest to compare our results with a recent work of Euler and Wässle (1998) on spatial distribution of GABA A and GABA C receptors on rat BCs. They recorded GABA receptor-mediated currents from BCs in the rat retinal slice preparation and found that the contributions of GABA A and GABA C receptors were related with the dichotomy of BCs respectively driven by rods and cones. That is, ~70% of the GABA current was mediated by GABA C receptors for rod BCs, while the fraction of the GABA C current was only ~20% for cone BCs. It should be noted, however, that a much lower standard GABA concentration (25 μM) was used in their work as compared with 1 mM used in the present work. For determining the actual receptor contribution, use of saturating concentration may have been required. In the present work, to examine the complements of GABA A and GABA C receptors on BCs, the saturating concentration (1 mM) of GABA was used to guarantee that all GABA receptors were fully activated. Since the GABA A and GABA C response components were completely suppressed by 0.5 mM BIC and 2 mM I4AA, respectively, in most cases, the complements of GABA A and GABA C receptors determined for a special subcellular site under such experimental conditions should reflect actual contributions of these two subtype receptors to the BCs.

The physiological implications of this differential distribution of GABA A and GABA C receptors on OFF and ON BCs remain to be explored. Here we only provide some speculations. First, because GABA C receptors have approximately a 10-fold higher sensitivity than GABA A receptors (for review,
GABAA receptors may be beneficial for ON BCs, which mediate inhibition from amacrine cells to BCs at low levels of GABA. On the other hand, predominant existence of GABA C receptors at BC terminals of a minority of BCs, their activation may be associated with illumination-dependent GABA levels in the synaptic clefts of the retina. It is generally thought that GABA is released from horizontal and amacrine cells in darkness and that the GABA release is reduced by illumination (Dowling 1987; Yazulla 1986). Since OFF BCs are activated when illumination is turned off, which corresponds to a change in GABA release from a lower to a higher level, the prevalent dominance of GABA C receptors on the axon terminals of these cells match such conditions, thus mediating inhibition from amacrine cells to BCs at low levels of GABA. On the other hand, predominant existence of GABA A receptors may be beneficial for ON BCs, which mediate inhibition in a relatively high background level of GABA since these cells are activated when light is turned on. Second, GABA C responses are overall slower than GABA A responses in kinetics. It has been shown that the time course of inhibition from amacrine cells to BCs may be differently shaped by these two receptor subtypes (Matthews et al. 1994; Pan and Lipton 1995). Finally, the differential subcellular localization of GABA A and GABA C receptors suggests that GABA C receptors (especially, of OFF BCs) may be mainly involved in visual signal processing in the inner retina, whereas GABA A receptors are in both the inner and outer retina.

Spatial distribution of receptors has been demonstrated to be crucial to their physiological functions (Magee et al. 1998; Nuesser and Somogyi 1997; Safronov 1999). In the CNS most neurons possess finer dendrites and axons; this makes such an analysis difficult. Moreover, since CNS neurons commonly have long processes, currents recorded from the soma could have been seriously decayed when drugs were locally applied to dendrites or axon terminals. In contrast, bullfrog BCs have a compact electrotonic architecture and their space constant (\( \lambda \)) ranges between 0.1 and 0.2 (unpublished observations). As a result, the axon terminal or dendritic GABA currents would decay no more than 10–20% when they were recorded from the soma (Spruston et al. 1993). Retinal BCs thus provide a good model for exploring physiological implication of spatial distribution of a special receptor type.

**GABA A receptors at BC axons**

When GABA was locally applied to BC axons, currents mediated by GABA A receptors were recorded from a population of BCs. It seems unlikely that these currents were induced by GABA diffusion to the dendrites of these cells, thus activating GABA A receptors on them. If it was the case, these currents should have been smaller than those recorded from the soma of the cell, a site that is closer to the dendrites. But we have consistently found that the results were reversed (see Fig. 8). Furthermore these currents were recorded whether or not the axon terminals were intact, suggesting that they were clearly not produced by activation of the GABA A receptors on the axon terminals of these cells due to diffusion of GABA locally applied to the axons. There are no data available concerning the existence of any GABA A receptor subunits on BC axons, but \( \rho \) subunits were recently found to be clearly clustered in synaptic hot spots along BC axons in mammalian retinae (Enz et al. 1996). These results suggest that BCs may receive GABAergic synaptic and/or extrasynaptic inputs along their axons.

Since there is no morphological evidence showing the existence of synapses on BC axons (Calkins et al. 1998), the GABA receptors found on BC axons must be extrasynaptic ones, which can be activated by the buildup of the bulk concentration of GABA in the retina following sustained activities of GABAergic cells (horizontal and amacrine cells). Modulation of BC signals mediated by the GABA receptors along the axons may be significantly different from that exerted by GABAergic inputs to the BC dendrites and axon terminals. It was reported in the CNS that GABA receptors at different subcellular sites may exert distinct physiological actions. For instance, in pyramidal neurons of hippocampus (Miles et al. 1996), the GABA A receptor-mediated inputs at the soma control the axonal outputs of the cells, whereas activation of the dendritic GABA A receptors suppresses calcium-dependent spikes originating from the dendrites. It sounds reasonable to speculate that the GABA A receptors along the BC axons may modulate signal conduction from the soma to the axon terminal. On the other hand, GABAergic inputs at the BC dendrites can strongly influence spatial and temporal integration of the glutamatergic postsynaptic potentials produced by photoreceptors (Du and Yang 1999a), whereas GABAergic inputs at BC axon terminals mainly modify transmitter release of BC (Dong and Werblin 1998; Hartveit 1999; Pan and Lipton 1995).

**Heterogeneity of GABA C receptors**

Three types of characteristic subunits of GABA C receptors \( \rho 1, \rho 2, \) and \( \rho 3 \) have been cloned in vertebrate retinas (for review, see Feigenspan and Bormann 1998). The multiplicity of \( \rho \) subunits and their splice variants suggest the existence of heterogeneous GABA C receptors. Evidence for this are the different PTX and/or I4AA sensitivities of native GABA C receptors in various animals documented in recent work. For instance, in fish, salamander, and ferret retinas, PTX can block completely the GABA C responses (Lukasiewicz 1996; Lukasiewicz and Wong 1997; Lukasiewicz et al. 1994; Qian and Dowling 1993, 1994), while GABA C currents of rat BCs were resistant to PTX (Feigenspan and Bormann 1993). Moreover, I4AA has been demonstrated to be a specific antagonist of GABA C receptors in a variety of retinas (Han et al. 1997; Kusama et al. 1993; Picaud et al. 1998; Qian and Dowling 1994, 1995) but was found recently to be able to activate GABA C receptors of BCs in the salamander retina (Lukasiewicz and Shields 1998b) and recombinant GABA C receptors of the white perch retina (Qian et al. 1998). In the present work, PTX could entirely block both the dendritic and axon terminal GABA C responses of the bullfrog BCs. For most of the cell tested, I4AA acted as a potent antagonist of GABA C receptors but had little effect on the GABA C receptors at the axon terminals of a minority of BCs, implying the existence of GABA C receptor heterogeneity, even in a single bullfrog BC.
REFERENCES


