Role of Neurotransmitter Receptors in Mediating Light-Evoked Responses in Retinal Interplexiform Cells

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Jiang Z, Shen W. Role of Neurotransmitter Receptors in Mediating Light-Evoked Responses in Retinal Interplexiform Cells. J Neurophysiol 103: 924–933, 2010. First published December 16, 2009; doi:10.1152/jn.00876.2009. Interplexiform (IP) cells are a long-neglected group of retinal neurons the function of which is yet to be determined. Anatomical study indicates that IP cells are located in the inner nuclear layer, juxtaposed with the third-order neurons. However, the synaptic transmission of IP cells in the inner retina is poorly understood. Using whole cell patch-clamp and pharmacological techniques, we extensively studied synaptic receptors in IP cells. The IP cells in amphibian retinal slices were identified by electrical and morphological properties with voltage-clamp recording and Lucifer yellow dialysis. We find that light-evoked excitatory postsynaptic currents (L-EPSCs) are mediated by AMPA and N-methyl-D-aspartate receptors in IP cells. Although both receptors contributed to the amplitude and kinetics of L-EPSCs, AMPA receptor desensitization substantially shaped L-EPSCs in the neurons, similar to those found in the third-order neurons. The light-evoked inhibitory postsynaptic currents (L-IPSCs) in IP cells were primarily mediated by strychnine-sensitive glycine receptors with a small component of GABA<sub>C</sub> receptors. GABA<sub>C</sub> receptor <p2 subunits were detected in IP cells with single-cell RT-PCR assays. Expression of GABA<sub>C</sub> receptors is one of the special features for IP cells, distinct from most of the third-order neurons that depend on GABA<sub>A</sub> and glycine receptors to relay the inhibitory signals. However, GABA<sub>A</sub> receptors in IP cells acted like nonsynaptic receptors that were activated by exogenous GABA application. Furthermore, L-IPSCs in IP cells were inhibited by the serial inhibitions between amacrine cells in the inner retina. In addition, application of neurotransmitters on the axon terminals of IP cells had no significant current generated in the cells, indicating that the synaptic inputs of IP cells are mainly from the inner retina. This study demonstrates the important role that light signals are encoded by both experiment of inhibitory receptors in IP cells.

INTRODUCTION

Interplexiform (IP) cells are a group of “long-range” feedback neurons involved in network regulations in the distal retinas. The morphology of IP cells show that the somas are located in the amacrine cell layer with their descending processes extending to the inner plexiform layer (IPL) and ascending processes extending to the outer plexiform layer (OPL); this creates a unique feedback loop from the inner retina to the outer retinas (Boycott et al. 1975; Djamgoz et al. 1991; Dowling and Ehinger 1978; Kolb and West 1977; Maguire et al. 1990; Mangel and Dowling 1985). Several types of IP cells have been identified in vertebrate retinas according to their putative neurotransmitter contents including dopamine, glycine, GABA, and somatostatin (Dowling and Ehinger 1975; Li et al. 1986; Marc and Liu 1984; Nakamura et al. 1980; Yang and Yazulla 1988). Among these cell types, dopaminergic IP cells have been extensively studied with respect to the regulations of photoreceptor synapses and horizontal cell gap-junction connection in light adaptation (Mangel and Dowling 1985; Schutte and Witkovsky 1991; Witkovsky et al. 1988). Our recent study of glycineric IP cells indicates that glycine feedback enhances synaptic transmissions in rod photoreceptors and horizontal cells (Shen 2005; Shen et al. 2008). Clearly, IP cell feedback plays a critical role in network modulation in the distal retina.

Although the output effects of IP cells have been investigated in the distal retinas, little is known about the synaptic inputs of the neurons. Previous studies from amphibian and goldfish retinas demonstrate that IP cells are transient neurons that respond to a light stimulus by brief depolarization at the onset and termination (Djamgoz et al. 1991; Maguire et al. 1990), indicating that IP cells receive glutamatergic inputs from ON and OFF bipolar cells in the IPL. This is similar to the synaptic inputs in the third-order neurons: amacrine and ganglion cells. The synapses within the IPL show that the excitatory signals are shaped by inhibitory GABAergic and glycinergic inputs from amacrine cells (Dong and Werblin 1998; Eggers and Lukasiewicz 2006; Zhang et al. 1997). However, it is unclear whether IP cells receive lateral inhibitory inputs from amacrine cells. Understanding the integration of synaptic inputs in IP cells will provide a better understanding of the function of the long-range feedback loop in the retinas.

The primary objective of this study is to determine the properties of synaptic inputs of IP cells in the retinal network. Our results indicate that light-evoked excitatory postsynaptic currents (L-EPSCs) in IP cells are mediated by both AMPA and N-methyl-D-aspartate (NMDA) glutamate receptors; the inhibitory signals in IP cells are primarily mediated by glycine receptors with a small component by GABA<sub>C</sub> receptors. The expression of GABA<sub>C</sub> subunits was detected in the amphibian IP cells. Our results demonstrate that IP cells integrate both excitatory and inhibitory inputs in a similar fashion as the third-order neurons.

METHODS

The present study was carried out with tiger salamander retinas, a highly accessible tissue for studying IP cells in whole cell recordings because IP cells in amphibian retinal neurons are large in size and have relatively high density in the retina. Importantly, the neural structure and function of salamander retinas are very similar to the higher vertebrates. IP cells in salamander retina share the general morphological characteristics to the neurons in other nonmammalian and mammalian retinas (Maguire et al. 1990). Specifically, the major group of IP neurons in salamander retinas is glycine- and somatostatin-containing cells without dopaminergic neurons (Li et al. 1986; Yang and Yazulla 1988).
Larval tiger salamanders (*Ambystoma tigrinum*), purchased from Kons Scientific (Germantown, WI) and Charles Sullivan (Nashville, TN), were used in this study. The animals were kept in aquaria at 11°C under a 12-h dark-light cycle with continuous filtration. The animals used were kept in ≥6 h in the dark. Briefly, the animals were quickly decapitated and double-pithed, and the eyes were dissected to remove the retinas. All procedures were performed in accordance with the guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University’s Animal Care Committee.

**Retinal slice preparation**

The retinal slices were prepared in a dark room, under a dissection microscope equipped with powered night-vision scopes (BE Meyer, Redmond, WA), an infrared illuminator (850 nm). An infrared camera and a video monitor were used to visualize the preparation. Briefly, a retinal tissue was removed from an eyecup in Ringer solution and mounted on a piece of microfilter paper (Millipore, Bedford, MA) with the ganglion cell layer downward. The filter paper with retina tissue was vertically cut at 250 μm/slice using a tissue slicer (Stoelting). A single retinal slice was placed in a recording chamber at a 90° angle to view the layers of the retina. The retinal slices were superfused with oxygenated Ringer solution, consisting of (in mM) 111 NaCl, 2.5 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 EGTA, and 10 dextrose, pH = 7.7. The recording chamber was placed on an Olympus BX51WI microscope equipped with a Rolera-MGiPlus CCD camera (Qimaging), linked to a monitor. An 850-nm infrared filter was used to block visible light from the light house of the microscope.

**Electrophysiological recording system**

Whole cell recordings were performed on IP cells and amacrine cells in dark-adapted retinal slices, using an EPC-10 amplifier and HEKA software. The patch electrodes (5–8 MΩ) were made with the MF-97 microelectrode puller (Sutter Instrument). The electrodes were filled with a high-potassium solution containing in (mM): 100 K-gluc OA108 and 5 EGTA, and 10 HEPES with 6U RNaseOUT, pH, 7.4. The cell content was extruded into a reaction tube kept in dry ice with aceton (−40°C). RT-PCR was performed immediately after the sampling. The SuperScript III one-step RT-PCR System was used for obtaining the RT-PCR products from the single-cell and whole tissue samples. The PCR primers were obtained from the Integrated DNA Technologies. The specific oligonucleotide primers were designed based on the cDNA sequences of human GABAC₂,β subunit: sense 5′-ACGACGCTGTCACCTACC-3′ and antisense 5′-AATG-GCATGG GTTGTCTGTG-3′. The cycling conditions were as follows: 50°C for 30 min, 94°C for 2 min followed by 45 cycles of 94°C 15 s, 55°C 20 s, and 68°C 30 s, followed by a final extension step of 68°C for 5 min. Unless otherwise specified, all the reagents and kits were purchased from Invitrogen (Carlsbad, CA). The RT-PCR products were cloned into pGEM T Easy vector (Promega, Madison, WI) and verified by DNA sequencing. The preliminary sequence was confirmed by two independent experiments. Single-cell RT-PCR methods were performed on three IP cells and two out of three had positive results. Also, four amacrine cells and one ganglion cell had been tested, and none of them showed positive results.

**RESULTS**

**Morphological and physiological profiles of IP cells in salamander retinas**

Whole cell recording was performed on dark-adapted retinal slices. To identify IP cells in retinal slices, Lucifer yellow (0.2%) was freshly added into the electrode solution before performing whole cell recordings. IP cells were located in the amacrine cell layer. Morphology of IP cells was revealed by Lucifer yellow dyes through whole cell recording electrodes, showing that the cell’s descending processes ramify at the on- and off-sublamina in the IPL, and a fine process from the soma ascends to the outer retina (Fig. 1A, see arrows right). These morphological features differentiated IP cells from amacrine and bipolar cells. Furthermore, whole cell voltage-clamp recordings indicate that IP cells have large voltage-dependent sodium currents (Fig. 1B, left), which are absent in bipolar cells and most amacrine cells. We used both criteria of morphology and electrophysiology to identify IP cells in retinal slices. A standard current-voltage (I-V) relationship curve indicated that IP cells had small inward-rectified currents and large outward K⁺ currents with both transient and sustained components, recorded from the voltage range of −100 to +60 mV as shown in Fig. 1B, right. From the IP cells (n = 57) recorded, the majority of the neurons had similar patterns of Na⁺ and K⁺ channel currents, as shown in Fig. 1B with the amplitudes of some cones. In some experiments, a bright light stimulus was also used to activate most of the cones in comparison with a dim light-evoked response. The duration of a light stimulus was 2–3 s.

**Single-cell RT-PCR assay**

The total ribonucleic acid (RNA) was extracted from two salamander retinas using the TRIzol reagent. To avoid RNase contaminations, the working surfaces were cleaned with RNase-zap (Ambion, Austin, TX). IP cells were identified by the morphology of the ascending axons labeled with Lucifer yellow and voltage-dependent Na⁺ currents in voltage-clamp recording. After IP cells were identified, the cell somas were gently pulled out from the slices. The cytoplasm of the somas was suctioned into a low resistance pipette that was filled with 3 μl intracellular solution containing (mM) 100 K⁺-gluc O108, 1 MgCl₂, 5 EGTA, and 10 HEPES with 6U RNaseOUT, pH, 7.4. The cell content was extruded into a reaction tube kept in dry ice with aceton (−40°C). RT-PCR was performed immediately after the sampling. The SuperScript III one-step RT-PCR System was used for obtaining the RT-PCR products from the single-cell and whole tissue samples. The PCR primers were obtained from the Integrated DNA Technologies. The specific oligonucleotide primers were designed based on the cDNA sequences of human GABAC₂,β subunit: sense 5′-ACGACGCTGTCACCTACC-3′ and antisense 5′-AATG-GCATGG GTTGTCTGTG-3′. The cycling conditions were as follows: 50°C for 30 min, 94°C for 2 min followed by 45 cycles of 94°C 15 s, 55°C 20 s, and 68°C 30 s, followed by a final extension step of 68°C for 5 min. Unless otherwise specified, all the reagents and kits were purchased from Invitrogen (Carlsbad, CA). The RT-PCR products were cloned into pGEM T Easy vector (Promega, Madison, WI) and verified by DNA sequencing. The preliminary sequence was confirmed by two independent experiments. Single-cell RT-PCR methods were performed on three IP cells and two out of three had positive results. Also, four amacrine cells and one ganglion cell had been tested, and none of them showed positive results.
these channel currents being variable from cell to cell. The typical current range for the peak Na\textsuperscript{+} currents were around a range of 400 pA to 1 nA. The different amplitude of Na\textsuperscript{+} currents could be due to loss of some Na\textsuperscript{+} channels in the dendrite or axon terminals of the cells in retinal slice preparation. Because IP cells possess voltage-dependent Na\textsuperscript{+} channels, light-evoked Na\textsuperscript{+}-dependent action potentials fire at the onset and termination of the stimulus (Fig. 1C). These are the general profiles of IP cells in the amphibian retina. Occasionally, some IP cells with dendritic processes asymmetrically distributed on one side of the soma were observed, but the physiological profiles of these cells were not significantly different from that of the major IP cells used.

**AMPA and NMDA glutamate receptors mediated light-evoked excitatory responses in IP cells**

To characterize excitatory synaptic inputs to IP cells, light-evoked postsynaptic currents (L-EPSCs) were recorded from the neurons in dark-adapted retinal slices. IP cells were voltage-clamped at −65 mV, which is close to the cell’s dark membrane potential. A dim light stimulus with a 3-s duration was used to evoke light responses in the neurons. The light stimulus caused a large, transient inward current at the onset, and a small inward current at the termination (Fig. 2A). The current amplitude of the offset light responses depends on the intensity and duration of the light stimulus. A large light offset response requires a bright stimulus with a longer "on" duration (data not shown), consistent with the light offset responses from amarine cells in a previous study (Yang et al. 2002). L-EPSCs, as shown in Fig. 2A, were predominantly glutamate-induced excitatory postsynaptic currents because IP cells were voltage-clamped at −65 mV, close to the Cl\textsuperscript{−} reversal potential (\(E_{Cl}\)) in the neurons. Possibly synaptic Cl\textsuperscript{−} current was near to zero at the \(E_{Cl}\). To specify which type of glutamate receptors were involved, selective receptor antagonists were used to block either AMPA or NMDA receptors. 1-(4-aminophenyl)-4-methyl-7,8-methylendioxy-5H-2,3-benzo-diazepine (GYKI) and D,L-2-amino-7-phosphonoheptanoic acid (AP-7) are selective antagonists for AMPA and NMDA.
receptors, respectively. With 30 μM GYKI, L-EPSCs in IP cells were reduced and the remaining currents were blocked by 80 μM AP-7; the L-EPSCs were partially recovered after washed with Ringer solution (Fig. 2B). These results indicate that the L-EPSCs in IP cells are mediated by both AMPA and NMDA receptors.

AMPA receptor desensitization is known to affect light-evoked responses in amacrine and ganglion cells (Tran et al. 1999). Therefore AMPA receptor desensitization would possibly limit the kinetics of L-EPSCs in IP cells. To study this, 100 μM cyclothiazide (CTZ) was applied to block AMPA receptor desensitization. As a control, 100 μM picrotoxin and 2 μM strychnine, the antagonists for GABA and glycine receptors were applied to eliminate the effects of network inhibition on IP cells. With picrotoxin and strychnine, CTZ increased the peak and duration of L-EPSCs in IP cells (Fig. 3A). On average, CTZ caused an increase of 23.6 ± 16.9% of the peak current (n = 4, P < 0.005). The amount of positive charge influx through AMPA receptors at the onset of a light stimulus was also calculated. CTZ caused a 95% increase of current flux through AMPA receptors compared with the control (n = 4, P < 0.001). The statistical results shown in Fig. 3B indicate that AMPA receptor desensitization could limit the duration of L-EPSCs in IP cells as found in amacrine and ganglion cells reported in the previous studies.

Furthermore, NMDA receptors were also found to contribute to the peak and kinetics of L-EPSCs in IP cells. IP cells were voltage-clamped at −30 mV. At this depolarizing voltage, the effect of magnesium blockage in NMDA receptors was removed. L-EPSCs were recorded from these IP cells in the presence of picrotoxin and strychnine. Application of AP-7 reduced the peak currents of L-EPSCs in the cells (Fig. 3C). The statistics from five tested IP cells indicated that AP-7 caused a 27.5 ± 12.3% decrease of the peak L-EPSCs (n = 5, P < 0.005), and the positive ions influx through NMDA receptors was reduced ~50.7 ± 19.5% of the control (n = 5, P < 0.001, Fig. 3D), suggesting that NMDA receptors also have a significant effect on the peak and duration of L-EPSCs in IP cells. Therefore the kinetics of L-EPSCs in IP cells are affected by both of AMPA receptor desensitization and NMDA receptor activation.

**Light-evoked inhibitory postsynaptic currents (L-IPSCs) in the IP cells**

A light stimulus can also activate amacrine cells that are either GABA- or glycineergic neurons and the inputs from amacrine cells evoke L-IPSCs in IP cells. To study GABA- and glycineergic inhibitory inputs, IP cells were voltage-clamped at 0 mV near the predicted reversal potential of glutamate-mediated L-EPSCs. GABA- and glycineergic inputs should cause outward currents at 0 mV. The results showed that transient outward currents were evoked at the on- and offset of a light stimulus, indicating that IP cells receive inputs from amacrine cells. L-IPSCs in IP cells have not been reported before. The components of L-IPSCs were studied by using potent GABA and glycine receptor antagonists. Figure 4A shows the results recorded at the onset of L-IPSCs. Strychnine (1 μM) was applied to block glycine receptors, which caused a significant reduction of L-IPSCs in IP cells. The remaining L-IPSCs were fully blocked by 10 μM imidazole-4-acetic acid (I4AA), a potent GABAC receptor inhibitor (Gao et al. 2000; Yang et al. 1999). Interestingly, the I4AA-sensitive component of L-IPSCs was insensitive to 30 μM SR95531, a potent GABA receptor antagonist. Figure 4B demonstrates that even with a high concentration of SR95531, strychnine still could not block the small L-IPSCs (the gray trace), but the currents were blocked by picrotoxin, a nonselective inhibitor that blocks glycine, GABA, and GABAC receptors in salamander retina (Shen and Slaughter 2001). In this case, picrotoxin blocked GABA receptors because both GABA and glycine receptors had already blocked by the high concentrations of SR95531 and strychnine. This ruled out the possibility that the small L-IPSCs were caused by insufficient doses of glycine and GABA receptor antagonists to block these receptors. GABA receptor contributions to L-IPSCs were also tested with different intensities of the stimulus because a high-intensity light stimulus could evoke a strong postsynaptic response that might activate receptors in the perisynaptic area. However, L-IPSCs evoked by both dim and bright stimuli were fully blocked by a combination of strychnine and I4AA/TPMPA (data not shown). The pharmacological results suggested that L-IPSCs in IP cells were mainly mediated by glycine receptors with a lesser extent of GABAC receptors; GABA receptors seemed to be uninvolved in L-IPSCs in the cells. The statistics show that strychnine alone blocked 62.4 ± 12.2% of the peak L-IPSCs at light-onset (n = 8, P < 0.0001), and the combination of strychnine and I4AA blocked nearly all of the L-IPSCs in all eight cells tested (P < 0.0001, Fig. 4C). These results suggest that the inhibitory receptors in IP cells are different from the
third-order neurons in which GABA$_A$ and glycine receptors predominantly mediate IPSCs.

Expression of GABA$_C$ p2 subunits was detected in IP cells in single-cell RT-PCR assays

To confirm that GABA$_C$ receptors are expressed in IP cells, single-cell RT-PCR assays were performed on total RNA in IP cell cytoplasm. GABA$_C$ receptors are comprised of heteromeric assemblies of p1–p3 subunits. For detecting GABA$_C$ subunits in the salamander retina, two pairs of primers were designed for each p subunit. The total RNA was extracted from single IP cells in retinal slices with low-resistance electrodes. Because GABA$_C$ receptors are expressed in bipolar cells, single bipolar cell and whole retinal tissue samples were used as positive controls. For negative controls, the reverse transcript enzyme was excluded from the reaction solutions. The expression of GABA$_C$ p2 was detected in single IP cells (Fig. 5A). The nucleotide sequence of the RT-PCR products from salamander IP cells have a high homology with that of higher vertebrates (human, 85.1%; mouse, 85.7%; and rat, 85.3%). The template protein sequence of GABA$_C$ p2 in salamander IP cells shows 93.7 and 94.3% homology to that of human and mouse. In addition, the protein sequences of GABA$_C$ p2 were compared between salamander amacrine cells, resulting in an increase of glycinergic input to IP cells. The serial inhibitions between GABA- and glycinergic amacrine cells have been reported from a previous study in salamander retina (Zhang et al. 1997). The L-IPSCs enhanced by glycine antagonists mimicked by SR95531 in IP cells (data not shown).

GABA$_A$ receptor antagonists enhanced L-IPSCs in IP cells

In this study, L-IPSCs were obtained to be small in IP cells in the control. Interestingly, the inhibitory currents largely increased when GABA$_A$ receptor antagonists were applied. Figure 6A shows that small L-IPSCs in IP cells were significantly increased when 100 $\mu$M bicuculline, a potent GABA$_A$ receptor antagonist, was applied in the bath solution. The effect of bicuculline was most likely to block GABA$_A$-mediated reciprocal inhibition on amacrine cells, resulting in an increase of glycinergic input to IP cells. The serial inhibitions between GABA- and glycinergic amacrine cells have been reported from a previous study in salamander retina (Zhang et al. 1997). The L-IPSCs enhanced by bicuculline were substantially reduced by 1 $\mu$M strychnine or 100 $\mu$M picrotoxin (Fig. 6A, bottom), indicating that blockage of GABA$_A$ inhibition increases glycinergic input to IP cells. This also suggests that GABA$_A$ inhibition in the network reduces L-IPSCs in IP cells. The data are also consistent with the results in Fig. 4 that strychnine-sensitive glycine response is the major cause of L-IPSCs in the cells. The remaining small L-IPSCs in the neurons should be a GABA$_C$ receptor-mediated current (see the outward currents in Fig. 6A, bottom), similar to the results as shown in Fig. 4A. The effect of bicuculline could be mimicked by SR95531 in IP cells (data not shown).

Picrotoxin (100 $\mu$M) blocked both GABA$_A$ and GABA$_C$ receptors in the system and also substantially increased L-IPSCs in IP cells, and the effect of picrotoxin was about twice as effective as bicuculline (Fig. 6B, middle, n = 13). With picrotoxin, strychnine completely blocked the L-IPSCs in the cells (Fig. 6B, bottom), further confirming that the small and sustained L-IPSCs in Fig. 6A were mediated by GABA$_C$ receptors. As picrotoxin had a stronger effect than bicuculline,
this raised the question of whether picrotoxin enhanced L-IPSCs by blockade of GABA_{C} inhibition in the network. According to previous studies in salamander retinas, GABA_{C} receptors in bipolar terminals inhibit glutamate release and picrotoxin blocks GABA_{C} receptors, resulting in an increase of glutamate release from bipolar cells (Dong and Werblin 1998; Lukasiewicz and Shields 1998). This could increase light-evoked excitation in amacrine cells that release glycine to IP cells. The effects of bicuculline and picrotoxin on light-evoked excitatory response in amacrine cells were examined and compared. Amacrine cells were voltage-clamped at −70 mV at which L-EPSCs were mainly glutamatergic currents. Our results showed that picrotoxin, but not bicuculline, was able to increase L-EPSCs in amacrine cells (Fig. 6, C and D), which is consistent with the results from the previous studies. Picrotoxin blocked network inhibition via both GABA_{A} and GABA_{C} receptors resulting in more glycinergetic inputs to IP cells. This indicates that both GABA_{A} and GABA_{C} receptors in the network contributed to suppression of L-IPSCs in IP cells.

GABA_{A} and GABA_{C} responses in IP cells

The majority of L-IPSCs in IP cells were mediated by glycine receptors, and the remaining small currents were mediated by GABA_{C} receptors. The pharmacological study suggests that GABA_{C} receptors may not effectively mediate L-IPSCs in IP cells. One could speculate if GABA_{A} receptors are present in IP cells in the somas or the extrasynaptic sites of the dendrites. To determine this, GABA was briefly applied at the inner retina where the dendrites and somas of IP cells are located. The network inputs were blocked with bath perfusion of 100 μM Cd^{2+} that has been commonly used to block Ca^{2+}-dependent synapses. GABA (100 μM) elicited an outward current in IP cells voltage-clamped at 0 mV. In the presence of 100 μM bicuculline, GABA-elicited currents were reduced. Because bicuculline is a potent GABA_{A} receptor antagonist, the bicuculline-sensitive currents could be GABA_{A} receptor–mediated currents. This suggests that bicuculline-sensitive GABA_{A} receptors are expressed in IP cells, possibly at the extrasynaptic areas of the cells. Moreover, with bicuculline, I4AA could further reduce GABA-elicited currents and the remaining currents were completely blocked by picrotoxin in the antagonist combination (Fig. 7A). We noticed that GABA-elicited bicuculline-insensitive currents in IP cells were found to have faster current decay kinetics compared with GABA_{C} currents in salamander bipolar cells. To explain the current decay kinetics, the time constant (τ) of the exponential decay was measured from the bicuculline-insensitive GABA-elicited currents in IP cells.
Light the time-to-peak rate of the bicuculline-insensitive currents the previous study (Lukasiewicz and Shields 1998). Moreover, the currents by bicuculline (* significantly blocked by strychnine ( receptor antagonist, bicuculline, largely increases L-IPSCs that are significantly reduced by bicuculline with 10 M I4AA and 100 M TPMPA could further reduce GABA-elicited currents to 52.2 ± 11.3% (n = 6, P < 0.005) and 41.5 ± 8.6% (n = 6, P < 0.001) of the control, respectively. The remaining currents were fully blocked by 100 M picrotoxin (n = 6, Fig. 7B). With pretreatment of bicuculline, both I4AA and TPMPA partially reduced GABA-elicited currents, but picrotoxin fully blocked the currents. This could be interpreted as GABA currents in IP cells have different sensitivity to these antagonists. I4AA and TPMPA might block the same type of GABA response, and those that were insensitive to these antagonists were blocked by picrotoxin.

Examining the effect of axonal inputs in IP cells

There is a possibility that axonal input in the distal retina might affect L-EPSCs and L-IPSCs in IP cells. Accordingly, glutamate, glycine, and GABA were applied at the OPL to mimic synaptic inputs at the axon processes of IP cells. The responses of the axon inputs were recorded from the somas of IP cells. Figure 8A shows the sketch of the experimental procedure. To depolarize the axon processes of IP cells, 100 mM potassium was locally applied in the OPL. The potassium was found to elicit EPSCs in IP cells voltage-clamped at −65 mV. The EPSCs were completely eliminated when the Ringer solution containing Cd was used (Fig. 8B). These results indicated that potassium-elicited EPSCs in IP cells were not a direct effect, but instead via bipolar cell synapses in the inner retina, because application of potassium in the OPL depolarized bipolar cells. Puffing glutamate at the OPL was found to have no effect on IP cells (data not shown). GABA or glycine was puffed on the axon processes of IP cells, while the neurons were clamped near the reversal potential of glutamate currents at 0 mV. With Cd in the Ringer solution, neither GABA nor glycine evoked currents in the axon processes of IP cells. However, when Cd was added to the bath solution, glutamate elicited currents in axon processes of IP cells that were sensitive to gabazine, bicuculline, and picrotoxin.

**Fig. 6.** Pharmacological study of L-IPSCs in IP cells. The cells were voltage-clamped at 0 mV. Blockage of network inhibition by a GABA receptor antagonist, bicuculline, largely increases L-IPSCs that are significantly blocked by strychnine (n = 5). B: picrotoxin blocks both GABA and GABA receptors in network, which significantly enhances L-IPSCs in an IP cell; with picrotoxin, the L-IPSCs are fully blocked by strychnine (n = 6). The small inward (downward) currents in the bottom trace are glutamate residue currents. C and D: the effects of bicuculline and picrotoxin on the L-EPSCs were recorded from amacrine cells held at −70 mV. Picrotoxin, but not bicuculline, substantially increases the L-EPSCs in amacrine cells. On average, the τ value of the currents was 499 ± 10 ms in IP cells, which is 10 times slower than the decay τ value of GABA currents obtained from salamander ganglion cells in the previous study (Lukasiewicz and Shields 1998). Moreover, the time-to-peak rate of the bicuculline-insensitive currents was −278 ms in IP cells, which is about five times slower compared to the time to peak of GABA currents in ganglion cells in the previous study. The τ values provide another line of evidence that the bicuculline-insensitive currents in IP cells might not be GABA currents in addition to the pharmacological evidence.

Another GABAC receptor antagonist, 1,2,5,6-tetrahydropyridine-4-yl-methylphosphinic acid (TPMPA), was used to block bicuculline-insensitive currents in IP cells. The effect of TPMPA was similar to I4AA that partially blocked the bicuculline-insensitive GABA currents. On average, bicuculline reduced GABA-elicited currents to 78.65 ± 5.4% of the control (n = 8, P < 0.005). With bicuculline, both 10 M I4AA and 100 M TPMPA could further reduce GABA-elicited currents to 52.2 ± 11.3% (n = 6, P < 0.005) and 41.5 ± 8.6% (n = 6, P < 0.001) of the control, respectively. The remaining currents were fully blocked by 100 M picrotoxin (n = 6, Fig. 7B). With pretreatment of bicuculline, both I4AA and TPMPA partially reduced GABA-elicited currents, but picrotoxin fully blocked the currents. This could be interpreted as GABA receptors in IP cells have different sensitivity to these antagonists. I4AA and TPMPA might block the same type of GABA response, and those that were insensitive to these antagonists were blocked by picrotoxin.

**Fig. 7.** GABA-elicited currents in IP cells are blocked by GABA and GABA receptor antagonists. IP cells were voltage clamped at 0 mV, and GABA was very briefly applied at the inner retina in slice preparation. A: puff GABA-elicited currents are reduced by bicuculline; the currents are further reduced by bicuculline with 10 M I4AA, and the currents are fully blocked by 100 M picrotoxin. B: the average suppressions of GABA-elicited currents by bicuculline (P < 0.005, n = 8), I4AA (***P < 0.001, n = 6), I4AA or 1,2,5,6-tetrahydropyridine-4-yl-methylphosphinic acid (TPMPA; NS: no significant difference, n = 6), and picrotoxin (****P < 0.001, n = 6). Error bar: SE.

**Fig. 8.** Study of the effects of potassium, GABA, and glycine at the axon processes of IP cells. A: the diagram of the experimental procedure. B: puff 100 mM potassium at the outer plexiform layer (OPL) elicits excitatory currents in an IP cell; the currents can be completely blocked by application of Cd in bath solution. C and D: in the presence of Cd, puff 100 mM GABA or 100 mM glycine on the OPL has generated no response in IP cells.
glycine found to be able to elicit a significant response in IP cells (Fig. 8, C and D). These results suggest that the inputs from the axon processes at the OPL did not significantly influence either the L-EPSCs or the L-IPSCs in IP cells. The results were repeatable (n = 5). Although the results suggest that the signals contributed from the axon processes were negligible, one still cannot rule out the possibility of GABA and glycine receptors being present in the distal axon processes of IP cells because the cable properties of long axon processes might limit electrical signal propagation to the somas of the cells.

**DISCUSSION**

By directly recording light-evoked responses from IP cells in dark-adapted retinal slices, the excitatory and inhibitory receptors in IP cells were investigated. The results agree with the previous studies that IP cells receive glutamatergic inputs and transiently depolarize to the onset and offset of a light stimulus (Djamgoz et al. 1991; Maguire et al. 1990). Our results further demonstrated that both AMPA and NMDA subtypes of glutamate receptors mediate L-EPSCs in IP cells. In addition, L-IPSCs were found to be primarily mediated by glycine and GABA<sub>B</sub> receptors; GABA<sub>A</sub> receptors were unlikely to be directly involved in synaptic L-IPSCs; instead the receptors acted more like extrasynaptic receptors activated by GABA local application. As shown that the synaptic inputs from the OPL had very little effect on the cell membrane potential, the action potentials in IP cells should be mainly triggered from the synaptic inputs from the IPL. Although it was observed that the dendrites processes of IP cells are ramified at different sublamina, the pharmacology of L-EPSCs and L-IPSCs in the neurons had no significant difference from cell to cell.

**Glutamate receptor subtypes in L-EPSCs**

Figure 3C indicates that inhibition of AMPA receptor desensitization by cyclothiazide can increase cation influx by ~95% in IP cells. A recent study indicates that cyclothiazide also blocks GABA<sub>C</sub> receptors (Xie et al. 2008). We believe that the increase in L-EPSCs produced by cyclothiazide was due to inhibition of AMPA receptor desensitization because GABA<sub>B</sub> receptor was blocked by PTX in our experiments; thus the fast desensitization of AMPA receptors might limit the light-evoked excitation in IP cells. We find that the properties of L-EPSCs in IP cells are similar to those in amacrine and ganglion cells with AMPA and NMDA glutamate receptors as major neurotransmitter receptors. The properties of ganglion cells are described as dim light-evoked EPSCs are mainly mediated by AMPA receptors, NMDA receptors are likely to mediate strong light-evoked EPSCs, and AMPA receptor desensitization shapes light responses in ganglion cells (Dixon and Copenhagen 1992). One may expect that glutamate receptors play a same role in light-evoked responses in IP cells.

**Inhibitory receptors in IP cells**

As shown, IP cells received both excitatory and inhibitory inputs in the inner retina; the function of inhibitory inputs is likely to shape L-EPSCs in IP cells. In addition, IP cells have widely extended dendritic processes that receive inhibitory inputs from surrounding amacrine cells. The lateral inhibition might suppress the excitatory response driven by a central light stimulus. This could create “center-surround” light response features in IP cells. Therefore the important function of inhibitory inputs can contribute to a spatial sensitivity in IP cells.

This study shows that the majority of L-IPSCs in IP cells are mediated by glycinerergic receptors, and a small part of L-IPSCs is mediated by GABA<sub>B</sub> receptors. These results are distinct glycinergetic IP cells from dopaminergic IP cells, in the latter the inhibitory inputs are mainly mediated by GABA<sub>A</sub> receptors (Feigenspan et al. 2000; Gustinich et al. 1999). IP cells receive synaptic inhibitory inputs in the IPL, like the third-order neurons. In fact, most inhibitory signals in the third-order neurons are mediated by GABA<sub>A</sub> and glycine receptors. There is controversial regarding whether GABA<sub>C</sub> receptors exist in the third-order neurons (Liu and Lasater 1994; Lukasiewicz et al. 2004; Rotolo and Dacheux 2003; Zhang and Slaughter 1995). Our study shows that GABA<sub>C</sub> receptor p2 subunits are expressed in IP cells via RT-PCR and GABA<sub>C</sub> responses are present in every IP cell tested in voltage-clamp recording, suggesting that IP cells are evidently distinct from the other third-order neurons.

So far, only two types of ionotropic GABA receptors, GABA<sub>A</sub> and GABA<sub>C</sub>, are identified in the CNS. In comparison with GABA<sub>A</sub> receptors, GABA<sub>C</sub> receptors are more sensitive to GABA, and GABA<sub>C</sub> currents are more sustained. Because GABA<sub>C</sub> receptors are insensitive to the potent antagonists of GABA<sub>A</sub> receptors, bicuculline and SR95331, these agents become the pharmacological tools to differentiate GABA<sub>A</sub> from GABA<sub>C</sub> receptor responses. GABA<sub>C</sub> receptors in retinas act as major feedback receptors in bipolar cell terminals in both mammalian and nonmammalian retinas (Eggers and Lukasiewicz 2006; Gao et al. 2000; Han et al. 1997; Lukasiewicz and Shields 1998; Pan and Lipton 1995). According to previous studies, both picrotoxin- and I4AA-sensitive GABA<sub>C</sub> receptors are present in salamander bipolar cells (Gao et al. 2000; Shen and Slaughter 2001; Yang et al. 1999), and TPMPA blocks picrotoxin-sensitive GABA<sub>C</sub> receptors (Shen and Slaughter 2001). In this study, the pharmacological profiles of GABA<sub>C</sub> receptors in IP cells show that the receptors are sensitive to I4AA, TPMPA, and picrotoxin. Furthermore, our results indicate that both I4AA and TPMPA effectively block synaptic GABA<sub>C</sub> responses in L-IPSCs but are less effective in blockade of bicuculline-insensitive currents when exogenous GABA is puffed on IP cells. On the other hand, picrotoxin effectively blocks GABA<sub>C</sub> currents elicited by either light-stimulation or GABA application. Thus from a pharmacological point of view, IP cells may have two types of GABA<sub>C</sub> responses, the I4AA/TPMPA-sensitive and the I4AA/TPMPA-insensitive responses. Both GABA<sub>C</sub> responses can be blocked by picrotoxin. Our results also suggest that the I4AA/TPMPA-insensitive GABA<sub>C</sub> receptors are most likely expressed at the somas and extrasynaptic sites of IP cells because I4AA fully blocked GABA<sub>C</sub>-mediated currents in L-IPSCs (see Fig. 4A). Although RT-PCR results show that GABA<sub>C</sub> p2 subunit is present in IP cells, it is also possible that other subunits, GABA<sub>C</sub> p1 and p3 may exist in IP cells. The results from pharmacological and molecular studies are consistent that IP cells possess GABA<sub>C</sub> receptors. It is worth noting that I4AA in higher concentrations also acts like an agonist for GABA<sub>A</sub> or GABA<sub>C</sub> receptors in salamander bipolar and ganglion cells in a previous study (Lukasiewicz and Shields 1998). However, the concentration of I4AA used in our experiments was 50 times lower than that used for the agonistic effect. I4AA (10 μM) has been used as a specific
antagonist for GABA<sub>C</sub> receptors in salamander retina (Gao et al. 2000; Yang et al. 1999). It seems that the desensitization rate of GABA<sub>C</sub> receptors in IP cells is faster than that of native or expressed GABA<sub>C</sub> receptors. In general, the decaying time constant for native GABA<sub>C</sub> currents is >1 s. The decay time constant of the I4AA/TPMPA-sensitive GABA<sub>C</sub> currents in IP cells was found to be ~499 ± 10 ms, which is about twice as fast as that of native GABA<sub>C</sub> receptors but 10 times slower than the decay time constant for GABA<sub>A</sub> currents in salamander retinal neurons. Because there is a large diversity of GABA<sub>C</sub> receptors due to alternative splicing (Martinez-Torres et al. 2004), this contributes to different desensitization rates of GABA<sub>C</sub> receptors in various cell types. In fact, a fast decay rate of GABA<sub>C</sub> response has been observed from carp retinal bipolar cells (Han et al. 1997). The fast desensitization of GABA<sub>C</sub> receptors in salamander IP cells observed in this study could be due to the receptor compositions with different p subunits or the subunit splicing variations. According to the studies on GABA<sub>A</sub> receptors, the receptor desensitization is regulated by intracellular protein-kinase-C- and cAMP-dependent phosphorylations (Huganir and Greengard 1990; Leidenheimer et al. 1992). The intracellular regulations could be another reason for the fast desensitization of GABA<sub>C</sub> receptors in IP cells. Nevertheless, our study indicates that GABA<sub>C</sub> receptors in IP cells have unique properties for determining the kinetics of light responses in neurons.

We find that the serial-inhibition between amacrine cells largely suppresses light-evoked glycinergic inputs to IP cells in the dark control conditions, and the suppression can be removed when bicuculline or picrotoxin are applied to block GABA<sub>A</sub> or GABA<sub>AAandC</sub> inhibitions in the network. This substantial network suppression on L-IPSCs of IP cells could be a reason why inhibitory synaptic inputs are not observed in a previous study (Maguire et al. 1990).

**General properties of IP cells in retinas**

This study demonstrates that IP cells have large Na<sup>+</sup> currents capable of generating action potentials. This feature allows electrical signals propagating through a fine axon to the terminal processes of IP cells in an orthogradic direction. Interestingly, we find that retrograde signals from the axon processes of IP cells had very limited effects on the cell membrane potentials. Possibly, there are only a few Na<sup>+</sup> channels in the axon terminal process that would limit the ability of action potentials to propagate in the retrograde direction in IP cells. It is also possible that the lack of both excitatory and inhibitory neurotransmitter receptors in the axon processes of the cells. Nevertheless, the special electrical properties let IP cells avoid neurotransmission derived from the outer retina. Using current techniques, we cannot perfectly elucidate whether there is any input in the axon processes of IP cells. A previous immunocytochemical study has demonstrated that horizontal cells have synaptic contacts on dopaminergic IP cells in fish retina (Marc and Lam 1981). This observation suggests that IP cells may receive an input from horizontal cells. According to our results, if horizontal cells make synapses on the axon processes of IP cells, the signals might be limited to the local regions and cannot reach to the soma by retrograde propagation. Therefore the net centrifugal feedback signal conducted through IP cells is a one-way direction: from the inner to the outer retina.

In conclusion, our study demonstrates the existence of both excitatory and inhibitory synaptic inputs in IP cells although the inhibitory inputs are suppressed by network inhibition in dark control conditions. The excitatory and inhibitory receptors were systematically characterized in IP cells. We also find that the input from outer retina has a very limited influence on the feedback neurons. This study documented the important properties of synaptic connections of glycinergic IP cells in amphibian retina.

**REFERENCES**


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