Activation of mGluR5 Modulates GABA<sub>A</sub> Receptor Function in Retinal Amacrine Cells

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Received 8 March 2002; accepted in final form 17 June 2002

Hoffpaur, Brian K. and Evanna L. Gleason. Activation of mGluR5 modulates GABA<sub>A</sub> receptor function in retinal amacrine cells. J Neurophysiol 88: 1766–1776, 2002; 10.1152/jn.00174.2002. Amacrine cells in the vertebrate retina receive glutamatergic input from bipolar cells and make synapses onto bipolar cells, ganglion cells, and other amacrine cells. Recent studies indicate that amacrine cells express metabotropic glutamate receptors (mGluRs) and that signaling within the inner plexiform layer (IPL) of the retina might be modulated by mGluR activity. This study tests the hypothesis that activation of mGluR5 modulates GABA<sub>A</sub> receptor function in retinal amacrine cells. Whole cell voltage-clamp recordings were combined with pharmacology to establish the identity of the ionotropic GABA<sub>A</sub> receptors expressed in primary cultures of chick amacrine cells and to determine how mGluR5 activity affected the behavior of those receptors. Application of GABA (20 μM) produced currents that reversed at −58.2 ± 0.9 mV, near the predicted Cl<sup>−</sup> reversal potential of −59 mV. The GABA<sub>A</sub> receptor antagonist, bicuculline (50 μM), completely blocked the GABA-gated currents. cis-4-Aminocrotonic acid (CACA; 100 μM), a GABA<sub>A</sub> receptor agonist, produced small currents that were not blocked by the GABA<sub>A</sub> antagonist, (1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic acid (TPMPA; 20 μM), but were completely blocked by bicuculline. These results indicate that cultured amacrine cells express GABA<sub>A</sub> receptors exclusively. Activating mGluR5 with (RS)-2-chloro-5-hydroxyphenylglycine (CHPG; 300 μM) enhanced GABA-gated currents by 10.0 ± 1.5%. Buffering internal Ca<sup>2+</sup> with BAPTA (10 mM) blocked the CHPG-dependent enhancement. Activation of PKC with the cell-permeable PKC activators (-)-7-octylindolactam V, phorbol 12-myristate 13 acetate (PMA), or 1-oleoyl-2-acyl-sn-glycerol (OAG) also enhanced GABA-gated currents in a dose-dependent manner. Pretreatment of PKC inhibited the mGluR5-dependent enhancement, and inhibition of Ca-dependent PKC isozymes with G66976 (35 nM) suppressed the effects of mGluR5 activation, suggesting that mGluR5 and PKC are part of the same pathway. To determine if mGluR5-dependent enhancement occurred at synaptic GABA<sub>A</sub> receptors, postsynaptic currents were recorded in the presence of CHPG. On average, the mean amplitudes of the quantal events were increased by about 18% when mGluR5 was activated. These results indicate that activation of mGluR5 enhances GABA-gated current in cultured amacrine cells in a manner that is both Ca<sup>2+</sup>- and PKC-dependent. These results support the possibility that glutamate released from bipolar cells can modulate the function of GABAergic amacrine cells and alter signaling in the inner plexiform layer.

INTRODUCTION

Amacrine cells are a morphologically and functionally diverse group of interneurons that mediate signaling in the inner plexiform layer (IPL) of the vertebrate retina. Although nearly 30 morphological classes of amacrine cells have been identified in the mammalian retina (MacNeil and Masland 1998; MacNeil et al. 1999), it is not yet clear how each class contributes to signal processing in the IPL. GABAergic amacrine cells, the focus of this investigation, are one of the most common types of amacrine cells in the vertebrate retina. GABAergic amacrine cells receive excitatory synaptic input from glutamatergic bipolar cells and inhibitory synaptic input from other GABAergic amacrine cells. These amacrine cells can make inhibitory synapses back onto bipolar cells, onto ganglion cells, and onto other amacrine cells (Dowling and Boycott 1965; Dubin 1970; Hartveit 1999). In the retina, synaptic input from bipolar cells depolarizes amacrine cells through the binding of glutamate to ionotropic glutamate receptors. The expression of metabotropic glutamate receptors (mGluRs) on postsynaptic amacrine cell processes in the mammalian retina (Cai and Pourcho 1999; Koulen et al. 1997) expands the role of glutamate into a possible modulator of amacrine cell function.

The mGluRs are an eight-member family of G protein-coupled receptors that are divided into three groups based on their sequence similarity and pharmacological properties (for review, see Conn and Pin 1997). Activation of group I mGluRs (mGluRs 1 and 5) leads to elevations in cytosolic IP<sub>3</sub> and Ca<sup>2+</sup>, and in some cells, to increases in cAMP (Aramori and Nakanishi 1992; Joly et al. 1995). Groups II (mGluRs 2 and 3) and III (mGluRs 4, 6, 7, and 8) are both negatively coupled to their sequence similarity and pharmacological properties (for review, see Conn and Pin 1997). Activation of group I mGluRs (mGluRs 1 and 5) leads to elevations in cytosolic IP<sub>3</sub> and Ca<sup>2+</sup>, and in some cells, to increases in cAMP (Aramori and Nakanishi 1992; Joly et al. 1995). Groups II (mGluRs 2 and 3) and III (mGluRs 4, 6, 7, and 8) are both negatively coupled to adenylyl cyclase, such that receptor activation reduces production of cAMP. Although it is well established that mGluR6 mediates synaptic transmission to ON bipolar cells, the role of other mGluRs in retinal function is poorly understood (Masu et al. 1995; Nakajima et al. 1993; Vardi and Morigiwa 1997; Vardi et al. 1998). In photoreceptors, activation of mGluR8 reduces the cytosolic Ca<sup>2+</sup> concentration, but the underlying mechanism has not been resolved (Koulen et al. 1999). In horizontal cells, activation of group I and III mGluRs causes enhancement of voltage-gated Ca<sup>2+</sup> currents (Linn and Gafka 1999), and activation of group III suppresses an inward rectifier current (Dixon and Copenhagen 1997). In the inner retina, activation of group III mGluRs on bipolar cell terminals reduces neurotransmitter release (Awatramani and Slaughter 2001) but enhances neurotransmitter release from amacrine cells (Caramelo...
et al. 1999). In ganglion cells, activation of group III mGluRs inhibits voltage-gated Ca^{2+} currents (Shen and Slaughter 1998). These studies indicate that mGluRs are present and functional in both the inner and outer retina. Nonetheless, much remains to be learned about the intracellular targets of activated mGluRs and the impact of mGluR activation on signal processing in the retina.

This study makes use of a culture system containing previously identified GABAergic amacrine cells (Gleason et al. 1993) and explores the role of a group I metabotropic glutamate receptor expressed by these cells. Previous studies established that GABAergic amacrine cells express mGluR5, and that Ca^{2+} elevations can be engendered by activation of these receptors (Kreimborg et al. 2001). Furthermore, the function of GABA{\textsubscript{A}} and GABA{\textsubscript{C}} receptors can be modulated by activation of PKC. In this study, the effects of mGluR5 activation are examined on the GABA-gated currents in individual amacrine cells and at their GABAergic synapses.

To begin to understand how activation of mGluR5 affects amacrine cell function, GABA-gated currents are recorded from individual amacrine cells using whole cell voltage-clamp techniques. Initially, the identity of the ionotropic GABA receptor expressed by these cells and the effects of mGluR5 activity on these receptors are examined. A pharmacological approach is then used to uncover some aspects of the downstream signaling mechanisms involved. Finally, to address whether modulation of GABA{\textsubscript{A}} receptors occurs at the synapse, we determine whether activation of mGluR5 alters the amplitude distribution of quantal events in amacrine cells. Our findings indicate that activation of mGluR5 enhances GABAergic signaling between amacrine cells by modulating the function of postsynaptic GABA{\textsubscript{A}} receptors and that PKC is a key component of the signaling pathway.

**Methods**

**Cell culture**

Chick retinal cultures were prepared as previously described (Gleason et al. 1992). Briefly, 8-day chick embryo retinas were dissociated in 0.1% trypsin and plated onto 35-mm plastic tissue culture dishes (Falcon, Oxnard, CA) that were previously treated with 1 mg poly-l-ornithine/ml (Sigma, St. Louis, MO). Cells were plated at a density of 1.25 × 10^5 cells/35-mm dish in Dulbecco’s modified eagle medium (Sigma) supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 1000 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM glutamine (Sigma). Cultures were fed every 2–3 days with Neurobasal-B-27 supplement (Gibco), 1000 U of penicillin/ml, 100 μg of streptomycin/ml, 10% fetal bovine serum (Hyclone, Logan, UT), 1 mM l-glutamine (Sigma, St. Louis, MO), and 100 μg/ml ascorbic acid (Sigma). Cultures were fed every 2–3 days with Neurobasal-B-27 supplement (Gibco), 1000 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM glutamine (Sigma). Cultures were incubated at 37°C under a 5% CO₂ atmosphere.

**Electrophysiology**

Electrophysiology experiments were performed on isolated amacrine cells 6–10 days after plating. Culture dishes were mounted on the stage of an Olympus IX70 inverted microscope equipped with Hoffman modulation contrast optics. Whole cell voltage-clamp recordings were made using an Axopatch 1-D amplifier, Digidata 1200 data acquisition board, and Clampex 7.0 software (Axon Instruments, Union City, CA). A reference Ag/AgCl pellet in 3 M KCl was connected to the culture dish via an agar bridge containing 3 M KCl. Patch electrodes were pulled from thin walled borosilicate glass (1.5 mm OD, 0.86 mm ID; Sutter Instruments, Novato, CA) using a Flaming/Brown Puller (Sutter Instruments). Tip resistance values were 5–10 MΩ for ruptured patch recordings and 3–5 MΩ for perforated patch recordings as measured in the bath. All recordings were made at room temperature (22–24°C).

To record whole cell GABA-gated currents, the ruptured patch technique was used with the standard internal solution (see Solutions). Before recordings were made, R{\textsubscript{M}} values were monitored and allowed to stabilize. Cells that exhibited unstable R{\textsubscript{M}} values during experiments were discarded from the data set. The measured liquid junction potential for the standard recording solutions was ~11 mV, and this value was applied to the voltage values in I-V plots. For recording quantal events, the perforated patch technique (Horn and Marty 1988) was used with amphoterocin B (Sigma) as the perforant. The pipette solution was prepared fresh every 1–2 h by making a 1:2 dilution of amphoterocin B (stock solution, 50 mg/ml DMSO) with Pluronic F-127 (stock solution, 25 mg/ml DMSO; Molecular Probes, Eugene, OR). This mixture was sonicated for 30 s and diluted in the perforated patch internal solution to a final concentration of 200 μg/ml.

**Solutions**

Unless otherwise indicated, reagents were purchased from Sigma. The standard extracellular solution contained (in mM) 116.7 NaCl, 5.3 KCl, 3.0 CaCl₂, 0.41 MgCl₂, 5.6 glucose, 3.0 HEPES, and 20 TEA-Cl. The pH was adjusted to 7.4 with NaOH. For experiments recording quantal events, the external solution contained 300 nM tetrodotoxin (TTX; Alomone Labs, Jerusalem, Israel) to block voltage-gated Na⁺ channels. The standard internal solution for ruptured patch recordings consisted of (in mM) 100 CsAc, 10 CsCl, 2 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, and 100 HEPES. The pH was adjusted to 7.4 with CsOH. The following ATP-regeneration reagents were added to the internal solution: 50 μM creatine phosphokinase, 3 mM ATP dipotassium salt, 1 mM ATP-dishodium salt, 20 mM phosphocreatine (Calbiochem, La Jolla, CA), and 2 mM GTP sodium salt. ATP-dipotassium and ATP-dishodium salts were prepared as 1 M and 300 mM stocks, respectively, and stored at −20°C. The intracellular recording solution for perforated patch recordings consisted of (in mM) 148.5 CsCl, 2 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, and 10 HEPES.

Stock solutions of (−)-7-octylindolactam V (Biomol Research Laboratories, Plymouth Meeting, PA), phorbol 12-myristate 13 acetate (PMA; Biomol), 1-oleoyl-2-acetyl-sn-glycerol (OAG; Biomol), and Gib6976 (Calbiochem) were prepared in DMSO and stored as single use aliquots at −20°C ≤ ±3 mo. When dissolved in external solution, the final DMSO concentrations did not exceed 0.1%, and control experiments showed that DMSO at that concentration did not affect GABA-gated currents (Fig. 4). GABA (Sigma) was routinely prepared as a 20 mM stock and stored at 4°C. 50 mM (RS)-2-chloro-5-hydroxyphenylglycine (CHPG; Tocris Cookson, Ballwin, MO) stocks were prepared in 100 mM NaOH, and stored at −20°C ≤ ±1 wk. The final pH of external solutions containing CHPG was readjusted to 7.4 with HCl. 100 mM cis-4-aminoacridonic acid (CACA; Tocris) and 20 mM (1,2,5,6-tetrahydropyridine-4-yl) methylphosphonic acid (TPMA; Tocris) stocks were prepared in water and stored at 4°C. Bicuculline methiodide, 15, 9(R) was dissolved directly in the external solution.

During all experiments, cells were under continuous perfusion at a rate of 1–2 ml/min. Rapid solution changes were achieved using a tri-barrel square glass assembly (0.6 mm ID, 0.1 mm wall; Warner Instruments, Hamden, CT) attached to a SF-77B Perfusion Fast Step (Warner Instruments) whose movement was controlled by the Clampex 7.0 software. Each barrel of the assembly was supplied by a six-to-one manifold (Warner Instruments). Manifold inlets were connected in various configurations to eight pressurized storage vessels. Solution flow from the individual reservoirs was manually controlled using the ValveLink 8 system (Automate Scientific, Oakland CA). GABA application was achieved in 10–20 ms through computer-controlled barrel movements. GABA-gated currents were routinely monitored using a current-clamp mode of the Clampex software. The intracellular pipettes were filled with an internal solution: 50 U/ml creatine phosphokinase, 3 mM ATP dipotassium salt, 1 mM ATP-dishodium salt, 20 mM phosphocreatine (Calbiochem, La Jolla, CA), and 2 mM GTP sodium salt. ATP-dipotassium and ATP-dishodium salts were prepared as 1 M and 300 mM stocks, respectively, and stored at −20°C. The intracellular recording solution for perforated patch recordings consisted of (in mM) 148.5 CsCl, 2 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, and 10 HEPES.
elicted by switching to GABA-containing external for 500 ms at 30-s intervals. All other solution changes were achieved in approximately 500 ms by opening and closing the valves upstream of a manifold feeding one common barrel. Control experiments demonstrated that solution switching did not alter GABA-gated current amplitude (Fig. 4A).

**Data analysis**

Changes in current amplitudes (as plotted in Figs. 2–7) were determined by calculating the difference between the peak GABA-gated current amplitude measured immediately before drug application and the peak current amplitude measured 30 s after the drug application. Quantal events were analyzed using MiniAnalysis (Synaptosoft Inc., Decatur, GA). All events detected by the software were visually inspected to ensure correct peak and baseline selection. Data are presented as the mean ± SE. Analyses of statistical significance were performed using either the unpaired (Figs. 2 and 3) or paired (Figs. 5–8) t-test.

**RESULTS**

**Cultured amacrine cells express GABA<sub>A</sub> receptors**

Amacrine cells were identified based on their morphology as previously described (Gleason et al. 1993). Individual amacrine cells voltage clamped at −70 mV produced an inward current in response to an external solution containing GABA. To determine the dose-response relationship, currents were measured in response to a range of GABA concentrations. A sigmoid fit to the data resulted in a calculated EC<sub>50</sub> of 50 ± 3 μM (Fig. 1A). To prevent desensitization that is observed at high concentrations, experiments were performed with 20 μM GABA. Voltage ramps delivered during GABA (20 μM) application produced currents that reversed at −58.2 ± 0.9 mV (n = 5), near the predicted Cl<sup>−</sup> reversal potential for the standard internal and external solutions (Fig. 1B). GABA-gated current rundown was consistently observed during the recording period (routinely about 10 min, Fig. 1C). Use of the perforated patch technique did not prevent the current rundown, suggesting that wash out of cytosolic components by the internal recording solution was not responsible for this rundown process.

To determine the identity of the ionotropic GABA receptors, whole cell GABA-gated currents were recorded in the presence of a GABA<sub>A</sub> receptor antagonist, bicuculline, or a GABA<sub>C</sub> receptor antagonist, TPMPA. In all cells tested (n = 9), bicuculline (50 μM) completely blocked the GABA-gated currents, suggesting that GABA<sub>A</sub> receptors are expressed (Fig. 1D). TPMPA (20 μM) reduced peak GABA responses by 10.5 ± 1.2% (Fig. 1E, n = 3), suggesting either that TPMPA interacted with GABA<sub>A</sub> receptors (Ragozzino et al. 1996) or that cultured amacrine cells expressed a small population of GABA<sub>C</sub> receptors. To further investigate the possibility of GABA<sub>C</sub> receptor expression, the effects of CACA (100 μM), a GABA<sub>C</sub> receptor agonist, were examined. CACA consistently evoked small inward currents (11.8 ± 1.5 pA; n = 11).
Bicuculline blocked the CACA responses (n = 5), but TPMPA was without effect (n = 8; Fig. 1, F and G). Thus the small effects of CACA were most likely due to interactions with a population of α6-containing GABA<sub>A</sub> receptors (Wall 2001, see Discussion). These results suggest that GABA<sub>B</sub> receptors are the only ionotropic GABA receptors expressed by cultured amacrine cells.

Amacrine cells can express metabotropic GABA<sub>B</sub> receptors (Catsicas and Mobbs 2001; Tian and Slaughter 1994), and it is possible that the steady change in GABA-gated current amplitudes (run-down) is due to slow modulation of the GABA<sub>A</sub> receptors by GABA<sub>B</sub> receptors. To address whether run-down was due to activation of GABA<sub>B</sub> receptors, we applied the GABA<sub>A</sub> receptor-specific agonist, isoguvacine (100 μM), in place of GABA. As for currents elicited with GABA, isoguvacine-dependent current amplitudes decreased over time (data not shown, n = 4). These results indicated that activated GABA<sub>B</sub> receptors were not responsible for the GABA<sub>A</sub> receptor current run-down observed in cultured amacrine cells.

Activation of mGluR5 enhances GABA-gated currents

To determine if activation of mGluR5 modulates GABA<sub>A</sub> receptor function, GABA-gated currents were recorded in the presence of CHPG, an mGluR5-specific agonist (Doherty et al. 1997). In 9 of 14 cells initially examined, application of CHPG (300 μM) enhanced GABA-gated currents by 10.0 ± 1.5% (Fig. 2). Throughout these experiments, CHPG modulated GABA-gated currents in about 66% of the cells. For subsequent experiments, the data are reported only for responding cells. Enhancement typically occurred within 30 s and persisted for the duration of CHPG exposure. We consistently observed that CHPG-enhanced GABA-gated currents had faster decay kinetics than control (Fig. 2B). Dose-response experiments showed that, in general, larger amplitude currents had faster decay rates, suggesting this effect was not necessarily a direct result of CHPG application. The rate of GABA-gated current run-down persisted in CHPG-treated cells. Voltage ramps delivered during GABA applications revealed no significant differences in the chloride reversal potential before (58.2 ± 0.8 mV) and during (57.2 ± 2.8 mV) CHPG application (data not shown; P = 0.655, n = 5). This result indicates that the effect of mGluR5 activation on the GABA-gated current amplitude is not due to a shift in the chloride reversal potential.

A previous study demonstrated that activating mGluR5 leads to increases in intracellular calcium in cultured amacrine cells (Kreimborg et al. 2001). To determine whether these rises in cytosolic calcium are required for the modulation of GABA-gated currents, BAPTA, a fast Ca<sup>2+</sup> chelator, was added to the standard internal solution to buffer the increases in intracellular calcium. For all cells examined, including BAPTA (10 mM) in the recording pipette blocked the current enhancement produced by CHPG (Fig. 3, A, B, and E; n = 5). Changes in normalized current amplitudes for BAPTA-treated cells following CHPG application averaged −2.0 ± 1.3% and were not significantly different (P = 0.916) from those observed in control cells (−1.6 ± 1.5%). Recordings made on the same day with the standard internal solution (1.1 mM EGTA) demonstrated that cells were responsive to CHPG if internal calcium buffering was reduced (Fig. 3, C and D; n = 2). These results are consistent with the modulation of GABA<sub>A</sub> receptor currents through a calcium-dependent pathway.

Activation of PKC enhances GABA-gated currents

Although an increase in intracellular calcium by activated mGluR5 has the potential to stimulate multiple effectors, the conventional downstream signaling pathway associated with mGluR5 involves the lipid- and calcium-dependent PKC isoforms. To determine if PKC activity enhanced GABA-gated currents in amacrine cells, GABA-gated currents were recorded in the presence of cell-permeable PKC activators. Within 30 s of application, (−)-7-octylindolactam V (250 nM) increased the mean current amplitude by 15.1 ± 1.8%, and current amplitudes increased up to 27.6 ± 2.5% during the 2-min application window (Fig. 4, A and B; n = 6). I-V plots showed that like CHPG, octylindolactam did not alter the reversal potential of GABA-gated currents (n = 4, data not shown). A diacylglycerol analog, OAG, and a phorbol ester, PMA, also reversibly enhanced GABA-gated currents in a dose-dependent manner (Fig. 4C). In most cases, the GABA-gated current enhancement achieved with activation of PKC
was considerably larger than that observed with activation of mGluR5. One possible explanation for this discrepancy is that only Ca\(^{2+}\)-dependent PKC isotypes are activated by the mGluR5 signaling pathway, whereas the PKC activators stimulate all subtypes of PKC. Alternatively, this discrepancy might indicate that the PKC activators stimulate a larger fraction of available PKC molecules.

Although 500 nM octylindolactam produced larger increases in GABA-gated current amplitudes, 250 nM octylindolactam was less toxic to the cells and allowed for experiments with longer application times. Recordings routinely became unstable during OAG and PMA applications, even at the lowest concentrations used. Therefore in most subsequent experiments, octylindolactam (at 250 nM) was used to activate PKC. Together, the results from the three PKC activators suggest that activation of PKC also leads to enhancement of currents through GABA\(_A\) receptors in amacrine cells.

**PKC activity is required for mGluR5-dependent modulation of GABA\(_A\) receptors**

To test whether mGluR5-dependent modulation of GABA-gated currents occurs via PKC activation, and if so, whether the mGluR5-dependent enhancement could be occluded, PKC was stimulated before activation of mGluR5. To accomplish this, octylindolactam was applied until a plateau in the PKC-dependent enhancement of GABA-gated current was achieved. CHPG was then applied in the continued presence of octylindolactam. When CHPG was applied to a cell that had been prestimulated with octylindolactam, no additional enhancement was observed (Fig. 5; \(n = 7\)). Both CHPG (300 \(\mu\)M) and octylindolactam (250 nM) independently enhanced GABA-gated currents by 8.4 \(\pm\) 1.4\% and 20.7 \(\pm\) 3.2\%, respectively.

If PKC activity is critical for the mGluR5-dependent enhancement of GABA-gated currents, then inhibiting PKC activity should block this effect. Exposure of the general PKC inhibitors, such as staurosporine (150 nM), sphingosine (3 \(\mu\)M), and calphostin C (2.5 and 0.5 \(\mu\)M), caused the recordings to become unstable. Interestingly, \(Ca^{2+}\) imaging experiments performed with the \(Ca^{2+}\) indicator fluo-3 revealed large and usually irreversible increases in cytosolic calcium during exposure to sphingosine consistent with cell damage (unpublished observations). G\(\delta\)6976, a selective inhibitor of Ca-depent isotypes of PKC, was less toxic than other PKC inhibitors when used at 25–35 nM, a concentration slightly above its reported EC\(_{50}\) value of 7.9 nM (Martiny-Baron et al. 1993). Application of G\(\delta\)6976 by itself enhanced GABA-gated currents by 3.3 \(\pm\) 1.4\% (\(n = 20\)). This finding was unexpected, given the enhancement observed with the PKC activators. Application of other general PKC inhibitors also enhanced GABA-gated currents before their toxic effects were observed. The exact source of this effect is unknown but may be attributable to the inhibition of basal PKC activity.
To establish that Go\(^6\)976 inhibited PKC-dependent GABA-gated current enhancement, currents were recorded while octylindolactam was applied alone and in the presence of Go\(^6\)976. Octylindolactam (100 nM) alone enhanced GABA-gated currents by 13.25 ± 2.49%, \((n = 8)\), and Go\(^6\)976 alone significantly enhanced GABA-gated currents by 1.55 ± 1.18%. Preapplication of Go\(^6\)976 significantly suppressed the effects of octylindolactam \((P = 0.016)\), reducing the current enhancement to only 5.08 ± 0.94% \((Fig. 6)\).

To determine whether inhibition of PKC also inhibits the mGluR5-dependent enhancement of GABA-gated current, the effects of G66976 on CHPG responses were examined. In co-application experiments similar to those described previously, activation of mGluR5 with CHPG (300 nM) alone enhanced the GABA-gated currents by 13.3 ± 3.3% \((n = 5)\). After washing out the CHPG, applying G66976 (35 nM) alone also enhanced the currents, in this subset of cells, by 8.2 ± 3.3% \((n = 5)\). However, no CHPG-dependent enhancement was observed in the presence of the inhibitor. Importantly, CHPG-dependent enhancement was restored after washing out the G66976 \((Fig. 7)\). Together, these results suggest that acti-vated mGluR5 enhances whole cell GABA-gated currents via activation of PKC in cultured amacrine cells.

**CHPG increases mean amplitude of quantal events**

Isolated amacrine cells form GABAergic synapses onto themselves, or autapses, after 9–10 days in culture, as previously described \((Frerking et al. 1995; Gleason et al. 1994)\). Thus it was possible to ask whether activation of mGluR5 modulated postsynaptic GABA\(_A\) receptors specifically. Using perforated patch voltage-clamp recordings to minimize wash out of synaptic activity, quantal release was promoted at autapses by holding the cell’s membrane potential between –40 and –50 mV, near the foot of the activation curve for voltage-gated calcium currents \((Gleason et al. 1994)\). To provide better resolution of the quantal events, a high Cl\(^–\) internal solution was used that gave a predicted \(E_{Cl}\) of 0 mV.

In agreement with previous recordings of synapses between pairs of amacrine cells \((Gleason et al. 1993)\), the amplitude distribution of quantal events recorded was positively skewed and exhibited some very large and rare events \((Fig. 8B)\). Application of CHPG reversibly enhanced the mean peak amplitude of quantal events in five of six cells examined. For
the remaining cell, the mean peak amplitude was slightly, but nonsignificantly, increased ($P = 0.610$) in the presence of CHPG. On average, CHPG significantly increased the mean quantal current amplitude by 18% ($P = 0.014$, $n = 6$; control = 9.0 ± 0.8 pA, CHPG = 11.6 ± 0.8 pA; Fig. 8C). In three of the five responding cells, cumulative frequency curves from data collected in CHPG were shifted to the right over the full range of amplitudes (Fig. 8D, a and b). In the other two cells, the shift was biased toward larger events (Fig. 8Dc).

DISCUSSION

Our results indicate that cultured retinal amacrine cells express GABA$_A$, but not GABA$_C$, ionotopic GABA receptors. Furthermore, the activity of these GABA$_A$ receptors is enhanced by mGluR5 activation, and experiments with BAPA-loaded amacrine cells indicate that this effect is Ca$^{2+}$-dependent. Pharmacological experiments suggest that the mGluR5-dependent enhancement is mediated by PKC, and recordings of quantal events indicate that mGluR5 activation specifically enhances currents through postsynaptically localized GABA$_A$ receptors. Together, these observations are consistent with glutamate release from bipolar cells acting as a modulator of amacrine cell function.

Modulation of GABA-gated currents by PKC

Activation of PKC was shown to modulate GABA$_A$ receptors in other systems, but the outcomes of the modulation have been variable (for review see Moss and Smart 1996; Swope et al. 1999). Previous studies on both native and heterologously expressed receptors demonstrated that pharmacological activation of PKC produces inhibition of GABA$_A$ receptor-mediated currents (Brandon et al. 2000; Connolly et al. 1999; Filippova et al. 2000; Gillette and Dacheux 1996; Kellenberger et al. 1992; Krishek et al. 1994; Leidenheimer et al. 1992; Sigel et al. 1991; Tapia et al. 1997). This inhibition was attributed to either direct phosphorylation of receptor subunits (Brandon et al. 2000) or to alterations in the levels of GABA$_A$ receptor surface expression (Connolly et al. 1999; Filippova et al. 2000).

In contrast to these reports of PKC-dependent inhibition of GABA-gated currents, intracellular delivery of constitutively active PKC subunits enhanced GABA$_A$ receptor-mediated currents through recombinant receptors expressed in mouse L929 cells (Lin et al. 1994, 1996). Similarly, addition of constitutively active PKC subunits into rat dentate gyrus cells also increased the conductance of GABA$_A$ receptor-mediated synaptic currents (Poisbeau et al. 1999). Although these results implied that the method of PKC activation (pharmacologically vs. introduction of active subunits) correlated with the outcome of GABA$_A$ receptor modulation, our results showing enhancement of GABA-gated currents after pharmacological activation of PKC argue against this scenario. Instead, the different modulatory effects of PKC on GABA-gated currents are likely to be due to factors intrinsic to different neuronal cell types. In support of this, Poisbeau et al. (1999) demonstrated that introducing constitutively active PKC subunits into dentate gyrus granule cells enhanced GABA$_A$ receptor function, but had no effect in CA1 pyramidal cells.

The different modulatory effects of PKC may be due to the heterogeneity of GABA$_A$ subunit expression in different neuronal populations. The binding patterns of subunit-specific antibodies in the IPL of the mammalian retina indicate that GABA$_A$ receptors can be assembled in a variety of subunit combinations (Greferath et al. 1995; Wässle et al. 1998). Although the full complement of GABA$_A$ subunits expressed by these GABAergic amacrine cells is currently unknown, two pieces of evidence suggest that cultured amacrine cells express $\alpha_6$ subunits. Wall (2001) demonstrated that GABA$_A$ receptors containing $\alpha_6$ subunits produced CACA-dependent currents that were insensitive to TPMPA. Thus our finding that bicuculline-sensitive and TPMPA-insensitive currents were elicited by CACA in these cells is consistent with the expression of the $\alpha_6$ subunit. GABA-gated currents in cultured chick amacrine cells were found to be sensitive to furosemide (S. Borges and M. Wilson, personal communication), an agent known to selectively inhibit GABA-gated currents in $\alpha_6$-expressing cells (Korpi et al. 1995; Wafford et al. 1996; Thompson et al. 1999).
Additionally, two novel GABA<sub>α</sub> subunits, β4 and γ4, were identified and sequenced from chick whole brain cDNA libraries (Bateson et al. 1991; Harvey et al. 1993). These subunits are capable of forming functional channels (Forster et al. 2001; Liu et al. 1998), but little is known concerning their modulation by PKC. Although functional expression of the β4 and γ4 subunits in the retina has not yet been demonstrated, in situ hybridization revealed that mRNA for the γ4-subunit is present in the inner nuclear layer of the retina, suggesting that a population of amacrine cells express this novel GABA<sub>α</sub> subunit (Harvey et al. 1994). Further elucidation of the subunit expression pattern in amacrine cells may shed light on the

FIG. 7. CHPG-dependent enhancement is inhibited by Go<sub>6976</sub>. A: peak current amplitudes of GABA-gated currents are plotted over time for a representative amacrine cell. CHPG (300 μM) and Go<sub>6976</sub> (35 nM) both enhance GABA-gated currents. In the presence of Go<sub>6976</sub>, CHPG does not enhance GABA-gated currents. Washing out the Go<sub>6976</sub> restores the CHPG-dependent enhancement. Applying Go<sub>6976</sub> during CHPG application reduces the mGluR5-dependent enhancement of GABA-gated currents. B: voltage-clamp recordings (denoted in A) before (a and f) and during (b and g) CHPG application, before (c) and during (d) Go<sub>6976</sub> application, and during co-application of CHPG and Go<sub>6976</sub> (e and h). C: CHPG-dependent enhancement of GABA-gated currents is suppressed when PKC is inhibited by Go<sub>6976</sub> (n = 6; *P = 0.02).

FIG. 8. CHPG increases the mean peak amplitude of GABAergic quantal events in isolated amacrine cells. A: representative recording of autaptic currents in an isolated amacrine cell in culture. B: amplitude distributions show that CHPG application reversibly increases the mean peak amplitude of the GABAergic quantal events in an amacrine cell. Data were collected for 90 s before, during, and after CHPG (300 μM) application. C: averages of the mean quantal amplitude data from 6 cells are plotted. CHPG significantly increased the mean peak amplitude by 18%. (*P = 0.01). D: cumulative frequency plots of quantal events recorded from individual amacrine cells before (dark trace) and during (light trace) CHPG application. The curves shown in a and b are positively shifted over the full range of amplitudes in the presence of CHPG. The data shown in A and B are from the same cell as the data shown in a. In the 3rd cell shown (d), the shift occurred primarily at larger amplitudes.
mechanisms underlying the effects of PKC on GABA<sub>Λ</sub> receptors.

**Role of mGluR5 at amacrine cell synapses**

The finding that activated mGluR5 enhances GABA-gated postsynaptic currents in cultured GABAergic amacrine cells indicates that, in the retina, glutamate might modulate synaptic interactions between amacrine cells. Activation of mGluRs was previously shown to affect GABAergic signaling at central synapses, but in each case the modulation targeted presynaptic mechanisms (Stefani et al. 1994; Gereau and Conn 1995; Poncer et al. 1995, 2000; Schrader and Tasker 1997; Semyanov and Kullmann 2000). The mGluR5-dependent enhancement of mean quantal event amplitudes suggests that a postsynaptic mechanism is targeted in amacrine cells. This interpretation is supported by our observation that whole cell GABA-gated currents are also enhanced. Interestingly, the percent of current enhancement of the quantal events is almost twice that observed for whole cell currents. There are two possible interpretations of this observation. It may be that the components of the signaling apparatus that sub-serve the effects of mGluR5 activation are postsynaptically (with respect to incoming GABAergic synapses) localized to preferentially target synaptic GABA<sub>Λ</sub> receptors for modulation. There is precedence for such an arrangement where β<sub>2</sub> adrenergic receptors, components of their signaling pathway, and their targets (L-type Ca<sup>2+</sup> channels) are co-localized by virtue of interactions with cytoskeletal elements (Davare et al. 2001). It has also been demonstrated that GABA<sub>Λ</sub> β subunits can be physiologically associated with PKC (Brandon et al. 1999).

The second scenario is that activation of mGluR5 also has an effect on presynaptic function. Multiple potential targets can be envisioned. One possibility is that at the synapse, activation of mGluR5 also augments filling of vesicles such that an enhancement in current amplitude is due to both changes in receptor behavior and the presence of a higher concentration of GABA. Another possibility is that mGluR5-dependent Ca<sup>2+</sup> elevations increase the frequency of exocytosis and may generate simultaneous vesicle fusions that would be detected postsynaptically as a single, large quantal event (Llano et al. 2000). At present, we think this is unlikely because activation of mGluR5 in the absence of voltage-gated Ca<sup>2+</sup> channel activation has not been observed to stimulate exocytosis in this preparation. It remains possible, however, that mGluR5-dependent release of Ca<sup>2+</sup> from internal stores, together with voltage-gated Ca<sup>2+</sup> channel activation would be sufficient to trigger simultaneous fusion events. Simultaneous events such as these might explain the increase in large amplitude quantal events that we observed in two of the six cells examined (see Fig. 8Dc).

How might the enhancement of GABAergic synaptic transmission affect signaling in the inner retina? One simple scenario would involve an amacrine cell process that is postsynaptic to a bipolar cell and also postsynaptic to a GABAergic amacrine cell. In this configuration, glutamate would enhance the efficacy of the synapse from the GABAergic amacrine cell and suppress the depolarization mediated by ionotropic glutamate receptors. This sort of anatomical arrangement has been described for the wide-field A22 amacrine cell of the cat retina (Kolb 1997). This cell is itself GABAergic and a single process can be postsynaptic to both bipolar cell and amacrine cell processes. Given the recent estimate that 92% of nonribbon synapses in the IPL are GABAergic (Marc and Liu 2000), it seems likely that contacts of this type frequently occur. These interactions would have the potential to be highly localized because of the nature (both serial and reciprocal) of amacrine cell synapses in the IPL.

It has been reported that bipolar cells can make ribbon synapses directly onto amacrine cell bodies (Bowd and Boycott 1965). Immunocytochemical evidence indicates that mGluR5 can also be expressed at cell bodies of amacrine cells in the intact retina (Kreimborg et al. 2001). Thus the Ca<sup>2+</sup> signaling engendered by activation of these receptors might alter amacrine cell function on a more global and long-term basis by activating Ca<sup>2+</sup>-dependent transcriptional regulation pathways. This type of transcriptional regulation was previously observed in rat striatal neurons, where activation of group I mGluRs led to phosphorylation of the MAP-K signaling components, ERK and Elk-1, as well as the transcriptional activator, cAMP response element-binding (CREB) protein (Choe and Wang 2001; Mao and Wang 2002). Understanding the degree to which activation of mGluR5 generates local signaling events in amacrine cells will be important in elucidating the full effect of glutamate on amacrine cell function and signaling in the IPL.

We thank J. Caprio and M. Wilson for critical reading of the manuscript. This work was supported by National Eye Institute Grant EY-12204 to E. Gleason and Sigma Xi Grant in Aid of Research to B. K. Hoffpauir.

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