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## Metabotropic glutamate receptor 5 and calcium signaling in retinal amacrine cells

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### Abstract

To begin to understand the modulatory role of glutamate in the inner retina, we examined the mechanisms underlying metabotropic glutamate receptor 5 (mGluR5)-dependent  $\text{Ca}^{2+}$  elevations in cultured GABAergic amacrine cells. A partial sequence of chicken retinal mGluR5 encompassing intracellular loops 2 and 3 suggests that it can couple to both  $G_q$  and  $G_s$ . Selective activation of mGluR5 stimulated  $\text{Ca}^{2+}$  elevations that varied in waveform from cell to cell. Experiments using high external  $\text{K}^+$  revealed that the mGluR5-dependent  $\text{Ca}^{2+}$  elevations are distinctive in amplitude and time course from those engendered by depolarization. Experiments with a  $\text{Ca}^{2+}$ -free external solution demonstrated that the variability in the time course of mGluR5-dependent  $\text{Ca}^{2+}$  elevations is largely due to the influx of extracellular  $\text{Ca}^{2+}$ . The sensitivity of the initial phase of the  $\text{Ca}^{2+}$  elevation to thapsigargin indicates

that this phase of the response is due to the release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum. Pharmacological evidence indicates that mGluR5-mediated  $\text{Ca}^{2+}$  elevations are dependent upon the activation of phospholipase C. We rule out a role for L-type  $\text{Ca}^{2+}$  channels and cAMP-gated channels as pathways for  $\text{Ca}^{2+}$  entry, but provide evidence of transient receptor potential (TRP) channel-like immunoreactivity, suggesting that  $\text{Ca}^{2+}$  influx may occur through TRP channels. These results indicate that GABAergic amacrine cells express an avian version of mGluR5 that is linked to phospholipase C-dependent  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  influx, possibly through TRP channels.

**Keywords:** (R,S)-2-chloro-5-hydroxyphenylglycine, amacrine cell, calcium, metabotropic glutamate receptor 5, transient receptor potential channels.

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Amacrine cells form a class of interneurons that mediate signaling in the inner retina. Although the exact function of most subtypes of amacrine cells is not known, their large number and diverse morphology suggest that amacrine cells are key players in retinal processing of the visual signal. Different subtypes of amacrine cells use a variety of neurotransmitters, including acetylcholine, glycine and GABA. GABAergic amacrine cells receive glutamatergic input from bipolar cells and make GABAergic synapses back onto bipolar cells and with other amacrine cells. Glutamate from bipolar cells can depolarize GABAergic amacrine cells by binding to ionotropic glutamate receptors and can potentially modify other aspects of amacrine cell function by activating metabotropic glutamate receptors (mGluRs). It has been demonstrated immunocytochemically that mGluRs can be expressed in the inner plexiform layer of the mammalian retina where amacrine cells extend processes and make synapses (Brandstätter *et al.* 1996; Koulen *et al.* 1996, 1997; Cai and Pourcho 1999). Furthermore, group I mGluRs (see below) have been specifically localized to

amacrine cell processes post-synaptic to bipolar cell terminals (Koulen *et al.* 1997; Cai and Pourcho 1999). We have previously demonstrated immunocytochemically that mGluR5 is expressed in the inner plexiform layer of the chicken retina and by identified GABAergic amacrine cells in chick retinal cultures (Kreimborg *et al.* 2001). These observations suggest that glutamate from bipolar cells can activate signaling pathways in, and modulate the function of, post-synaptic GABAergic amacrine cells.

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*Abbreviations used:* ACPD, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid; AUAP, abridged universal amplification primer; CHPG, (R,S)-2-chloro-5-hydroxyphenylglycine;  $\text{EC}_{50}$ , median effective concentration; fluo-3, AM, fluo-3 acetoxymethyl ester; G, GTP binding (protein);  $\text{IP}_3$ , 1,4,5-inositoltrisphosphate; mGluR, metabotropic glutamate receptor; PKC, protein kinase C; TRP, transient receptor potential.

The mGluRs constitute a family of G protein-coupled receptors that has been subdivided into three groups based on amino acid sequence [for a review see Conn and Pin (1997)]. Group I contains mGluR1 and mGluR5, group II contains mGluR2 and mGluR3 and group III contains mGluR4, mGluR6, mGluR7 and mGluR8. Cloned mammalian group I receptors examined in heterologous expression systems have been linked to phosphoinositide turnover. Stimulation of cAMP production has also been demonstrated (Aramori and Nakanishi 1992; Joly *et al.* 1995). Additionally, mutational analysis of cloned rat mGluR1 has identified a small number of amino acid residues in intracellular loops 2 and 3 that are required for the receptor's ability to interact selectively with  $G_q$  and  $G_s$  (Francesconi and Duvoisin 1998). Interestingly, this same amino acid sequence is also found in mGluR5, raising the possibility that both mGluR1 and mGluR5 can couple to  $G_q$  and  $G_s$ . Coupling to  $G_q$  would link these receptors to phospholipase C activation, liberation of 1,4,5-inositoltrisphosphate ( $IP_3$ ) and subsequent release of  $Ca^{2+}$  from internal stores. Coupling to  $G_s$  would link these receptors to adenylate cyclase activation and generation of cAMP that could activate protein kinase A and/or gate cyclic nucleotide-gated channels. Although the residues identified by Francesconi and Duvoisin (1998) confer the potential to couple to  $G_q$  and  $G_s$ , whether these interactions actually occur is apparently dependent upon the cell type (Abe *et al.* 1992; Selkirk *et al.* 2001).

Fairly little is known about the function of group I receptors in the vertebrate retina. With respect to the chicken retina, there is pharmacological evidence that (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD, group I and II agonist) stimulates phosphatidylinositol turnover in chick retinal cultures but no  $Ca^{2+}$  elevations were detected from whole cultures (Duarte *et al.* 1996). Similar measurements from individual cultured amacrine cells using more specific agonists, however, indicate that  $Ca^{2+}$  elevations can be elicited in amacrine cells by activation of group I receptors (Kreimberg *et al.* 2001). Another mechanism that could play a role in mGluR5 signaling is the activation of transient receptor potential (TRP) channels [for a review see Harteneck *et al.* (2000) and Clapham *et al.* (2001)]. TRP channels are a large (~20 in mammals) family of transmembrane proteins that are generally cation permeable and mediate  $Ca^{2+}$  entry under a variety of conditions. The gating of this family of channels is not well understood, but several regulators have been identified, including  $Ca^{2+}$  store depletion, diacylglycerol, activation of phospholipase C, and internal  $Ca^{2+}$ . The regulation of TRP channels by these particular molecules indicates that activation of the  $IP_3$  pathway and activation of TRP channels may often co-occur.

The expression and regulation of signaling pathways in retinal amacrine cells are poorly understood. Elucidating the linkage between activation of mGluR5 and downstream signaling mechanisms will improve our understanding of

these receptors in signal processing in the inner retina. To confirm the possibility that mGluR5 expressed in the chicken retina has the potential to couple to both  $G_q$  and  $G_s$ , we used a RT-PCR strategy to amplify a fragment of chicken mGluR5 encompassing intracellular loops 2 and 3. To examine some of the consequences of mGluR5-dependent signaling, we recorded relative  $Ca^{2+}$  concentrations from fluo-3 acetoxymethyl ester (fluo-3 AM)-loaded cultured GABAergic amacrine cells. To activate the receptor, we employed (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG), an agonist specific for rat mGluR5 (Doherty *et al.* 1997). Additionally, immunocytochemical methods were used to examine the expression of TRP channel subunits that may participate in generating mGluR5-dependent  $Ca^{2+}$  elevations.

## Materials and methods

### Materials

The molecular biology reagents included RNazol (Tel. Test Inc, Friendswood, TX, USA), Dnase I (Roche Molecular Biochemicals, Mannheim, Germany), 3' Race System (Gibco, Rockville, MD, USA), Failsafe Enzyme Mix (Epicentre Technologies, Madison, WI, USA). For  $Ca^{2+}$  measurements, the cells were loaded with fluo-3 AM (Molecular Probes, Eugene, OR, USA). CHPG was obtained from Tocris Cookson (Ballwin, MO, USA). The reagents U73122, U73343 and thapsigargin were purchased from Calbiochem (San Diego, CA, USA). For the electrophysiology experiments, nifedipine was obtained from Tocris Cookson, tetrodotoxin was obtained from Alomone Laboratories (Jerusalem, Israel), and amphotericin B was obtained from Sigma (St. Louis, MO, USA). Polyclonal primary antibodies raised against TRPC1, 3 and 6 were obtained from Alomone Laboratories and secondary antibodies were obtained from Chemicon (Temecula, CA, USA). All other reagents were obtained from Sigma.

### RT-PCR and sequencing

Retinal tissue was obtained from White Leghorn chickens (*Gallus gallus domesticus*) from the Poultry Science Department, Louisiana State University. The chickens were killed by cervical dislocation and decapitation. All methods employing live animals were approved by the Louisiana State University Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Retinas were removed and RNA was isolated with RNazol following the manufacturer's protocol. To ensure that all genomic DNA was removed, the samples were treated with Dnase I prior to the RT reactions. Negative controls without reverse transcriptase confirmed that genomic DNA was not a source of amplification.

The initial RT-PCR protocol and primers were designed to amplify and identify mGluR sequences between transmembrane segments II and V, as previously described (Medler *et al.* 1998). The gel-purified PCR product was cloned and sequenced using an ABI Prism 377 DNA sequencer using Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed and compared with previously published mGluR sequences using BLAST. GENE RUNNER software (Hastings

Software, Hastings, NY, USA) was used to obtain the predicted amino acid sequences.

To obtain sequence data downstream of transmembrane segment V, specific primers were designed for use with the 3' Race System. First-strand synthesis was performed with the supplied adapter primer (oligo dT) as per the manufacturer's protocol. Primers for the second-strand synthesis included the abridged universal amplification primer (AUAP) provided and a gene-specific primer (GSP) 5'-GAGGCTACTTGGATTGTAACACCACC. The GSP-primed PCR was initiated at 94°C with Failsafe Enzyme Mix and amplified according to the following schedule: 94°C for 1 min, 60°C for 1 min, 72°C for 3 min, for 35 cycles followed by 72°C for 10 min. A 1 : 100 dilution of the resulting product was used as the template for amplification with a nested primer, 5'-CTTGGATACAATGATTATTAATATTGAG and the AUAP. The nested PCR was initiated at 94°C and amplified according to the following schedule: 94°C for 1 min, 55.2°C for 1 min, 72°C for 3 min, for 35 cycles followed by 72°C for 10 min. Identification of the amplification products was performed as described above.

### Cell culture

Fertile White Leghorn chicken eggs were obtained from Louisiana State University Poultry Science Department and were incubated in a humid 39°C chamber. Cultures were prepared from 8 day chick embryos, as previously described (Gleason *et al.* 1993). Amacrine cells were identified by their morphology (Gleason *et al.* 1993) and were used after 7–10 days in culture.

### Ca<sup>2+</sup> measurements

Measurements of the relative Ca<sup>2+</sup> concentration were carried out using previously described methods (Kreimborg *et al.* 2001). For these experiments, data were collected every 5 or 10 s. To determine whether peak Ca<sup>2+</sup> elevations for fast responses were being missed with our slow sampling rate, a subset of cells ( $n = 5$ ) was tested with pulses of high K<sup>+</sup> external (see below), measured at three sampling intervals (3, 5 and 10 s). We found that with a 3 s sampling interval there were from six to 10 data points within 90% of the true peak, indicating that with a 10 s sampling interval we may underestimate the true peak by 10% at most.

Relative response amplitudes varied from cell to cell. This variability was not related to the response waveform and could reflect cell to cell differences in dye loading or resting Ca<sup>2+</sup> levels. Differences in dye concentration can alter the time course of Ca<sup>2+</sup> elevations in cells (Borst *et al.* 1995). However, for a group of 15 cells where data acquisition values were identical, we found that differences in baseline fluorescence levels were unrelated to the waveform of the Ca<sup>2+</sup> elevations. The observed variability should have no effect on our interpretations of these data because for all analyses, the response amplitudes and durations were normalized to control responses elicited in the same cell.

All solutions were delivered through gravity-driven bath perfusion (3–4 mL/min) with a 1 mm inlet positioned about 2–3 mm upstream from the cells. Using dye-containing external solution, we estimated that the dead space in the perfusion system accounted for about 10 s of the delay between switching on the agonist and the onset of the response. The external solutions were (in mM): normal external: KCl, 5.3; NaCl, 135.0; CaCl<sub>2</sub>, 3.0; MgCl<sub>2</sub>, 0.41; glucose, 5.6; HEPES, 3.0. High K<sup>+</sup> external: KCl, 100.0; NaCl, 15.0; CaCl<sub>2</sub>, 3.0; MgCl<sub>2</sub>, 0.41; glucose, 5.6; HEPES, 3.0. Ca<sup>2+</sup>-free external:

KCl, 5.3; NaCl, 135.0; MgCl<sub>2</sub>, 0.41; glucose, 5.6; HEPES, 3.0. The pH was adjusted to 7.4 with 1 M NaOH. A 10 mM stock of CHPG was prepared in 0.1 M NaOH and diluted to 50 μM in the appropriate external solution then the pH was adjusted to 7.4. Although the median effective concentration (EC<sub>50</sub>) value for CHPG in cloned and heterologously expressed rat mGluR5 was 750 μM (Doherty *et al.* 1997), we tested our cells with CHPG concentrations ranging from 50 to 300 μM and found no differences in responsiveness over this range. This difference in agonist efficiency may be due to species differences or may stem from the differences between heterologously expressed and native receptors. Analyses of the response amplitudes were carried out on raw data without normalization but after subtraction of baseline values.

### Electrophysiology

Perforated-patch whole cell voltage clamp recordings were made on individual isolated amacrine cells, as previously described (Gleason *et al.* 1994). Briefly, 2–4 MΩ resistance electrodes were filled with internal solution containing 200 μM amphotericin B. Within 5 min of seal formation, the series resistance values had usually fallen below 50 MΩ. Series resistance values were monitored throughout each recording and only cells with stable series resistance values were retained in the data set. The internal solution consisted of (in mM): Cs acetate, 135.0; CsCl, 10.0; NaCl, 1.0; MgCl<sub>2</sub>, 2.0; CaCl<sub>2</sub>, 0.1; EGTA, 1.1; HEPES, 10.0. The external solution consisted of (in mM): NaCl, 116.7; KCl, 5.3; Tetraethylammonium (TEA) Cl, 20.0; CaCl<sub>2</sub>, 3.0; MgCl<sub>2</sub>, 0.4; glucose, 5.6; HEPES, 3.0; and 300 nM tetrodotoxin to block voltage-gated Na<sup>+</sup> channels. The experiments were performed at room temperature (24–27°C).

### Immunocytochemistry

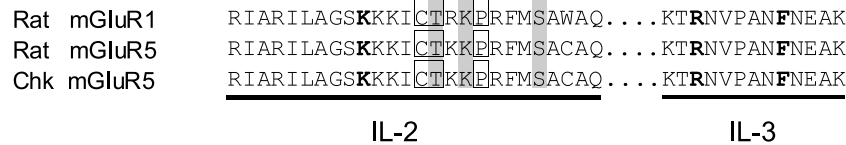
Anti-TRPC1, 3 and 6 are all polyclonal antibodies raised in rabbits against unique and conserved epitopes of TRP channel subunits cloned from humans (TRPC1) or mice (TRPC3, 6).

After 8 days in culture, dishes of chicken retinal cells from three different primary cultures were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at 4°C. Blocking solution and antibody dilutions were performed in dilution solution containing 1% bovine serum albumin and 0.5% saponin in PBS. The cultures were incubated in 5% normal goat serum for 1 h at room temperature. Primary antibodies were applied at 1 : 250 (2.4 ng/mL) dilutions for 1 h at room temperature. For experiments with blocking peptides, undiluted primary antibody was co-incubated with the appropriate antigen peptide at a ratio of 1 : 4 and incubated overnight at 4°C. These antibodies were diluted to 1 : 250 just prior to use. Following rinsing, Cy3-conjugated goat anti-rabbit secondary antibodies (1 : 1000) were applied for 1 h at room temperature, rinsed, then covered in a solution containing 27% PBS, 3% *n*-propyl gallate (w/v) and 70% glycerol.

## Results

### The chicken mGluR5 amino acid sequence indicates coupling to G<sub>q</sub> and G<sub>s</sub>

To determine whether the residues in intracellular loops 2 and 3 critical for mammalian mGluR1 coupling to G<sub>q</sub> and G<sub>s</sub> (Francesconi and Duvoisin 1998) are also found in chicken



**Fig. 1** Chicken metabotropic glutamate receptor 5 (mGluR5) contains conserved sequences for coupling to G proteins  $G_q$  and  $G_s$ . The amino acid sequences for intracellular loops (IL) 2 and 3 of chicken mGluR5 are aligned with rat group I mGluRs. The amino acids required for

coupling to  $G_q$  are in shaded boxes, while those residues involved in  $G_s$  coupling are in open boxes. Residues required for coupling to either G protein are shown in bold. The sequence information for rat mGluR1 and mGluR5 was adapted from Francesconi and Duvoisin (1998).

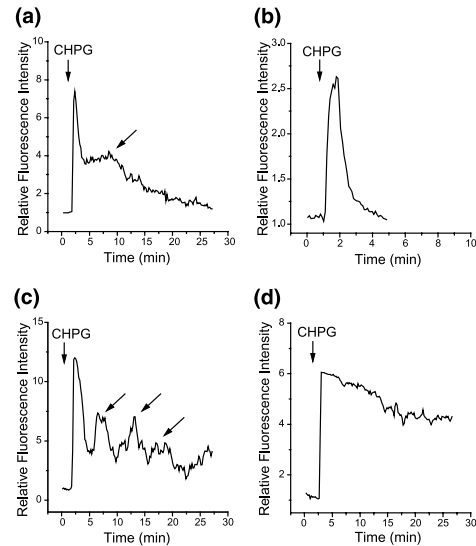
mGluR5, we used a RT-PCR strategy to generate a partial sequence (801 bp) of mGluR5 expressed in the chicken retina accession number AY078349. This sequence encompassed a region from the beginning of the second transmembrane domain through the first 45 amino acids of the C-terminus. Over this region, a comparison with rat mGluR5 showed that chicken mGluR5 was 84% similar at the nucleotide level and 99% similar at the amino acid level. We also found that the amino acids in the chicken mGluR5 intracellular loops 2 and 3 were identical to those found in rat mGluR1 and mGluR5 (Francesconi and Duvoisin 1998; Fig. 1). Although other regions of the receptor may also contribute to the selectivity of G protein coupling, these results suggest that chicken mGluR5 may have the potential to couple to both  $G_q$  and  $G_s$ .

#### Identification of amacrine cells

Cultured amacrine cells have large (10–15  $\mu\text{m}$  diameter) cell bodies and from two to five primary processes. Although there is some morphological diversity among amacrine cells in culture, they can easily be distinguished from the oil droplet-bearing cone cells (Adler *et al.* 1984; Gleason *et al.* 1992), putative bipolar cells (cell body diameter  $\sim 5 \mu\text{m}$ ; Gleason and Wilson, unpublished observations) and a third population of cells that bear a radial array of short processes and may be horizontal cells (Kreimborg *et al.* 2001). After 7–10 days in culture, the GABAergic amacrine cells have acquired numerous characteristics of adult amacrine cells, including expression of the appropriate ligand-gated ion channels (Huba and Hofmann 1991), voltage-gated ion channels (Huba *et al.* 1992) and functional GABAergic synapses (Gleason *et al.* 1993). On each of these physiological criteria, the amacrine cells in culture comprise a uniform population and in this study, we treated them as a single group.

#### Activation of mGluR5 elicits intracellular $\text{Ca}^{2+}$ elevations

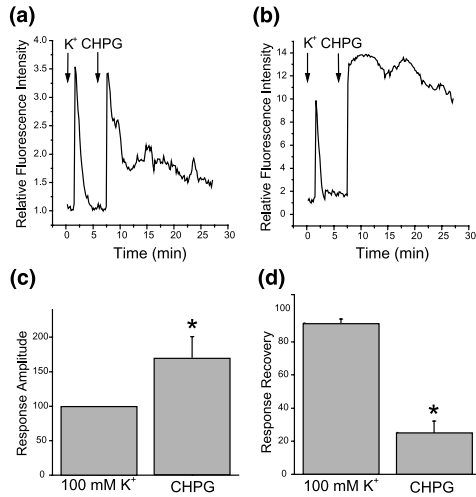
Cytosolic  $\text{Ca}^{2+}$  elevations were detectable in response to 50  $\mu\text{M}$  CHPG in 100% of amacrine cells examined ( $n = 42$ ). Although these cells were similar to each other on the criteria outlined above, different cells generated CHPG-dependent  $\text{Ca}^{2+}$  elevations with different temporal properties (Fig. 2).



**Fig. 2** (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG) elicits intracellular  $\text{Ca}^{2+}$  elevations. CHPG (50  $\mu\text{M}$ ) was added to the bath for 20 s at the time indicated by the vertical arrow. (a) A representative amacrine cell that showed an initial spike in cytosolic  $\text{Ca}^{2+}$  followed by a prolonged shoulder (arrow). (b) The response of a different amacrine cell to the same stimulation showed the spike but no shoulder (note the different time scale for this panel). (c) These data are representative of the amacrine cells that responded with a spike of  $\text{Ca}^{2+}$  elevation followed by a period of  $\text{Ca}^{2+}$  oscillations. (d) Data are shown for an amacrine cell where a prolonged  $\text{Ca}^{2+}$  elevation was elicited.

The  $\text{Ca}^{2+}$  elevations could be classified into four groups. Seventeen percent of cells examined responded with an initial spike, followed by a prolonged shoulder (Fig. 2a), while 29% of cells responded with a spike but failed to display the shoulder (Fig. 2b). Other amacrine cells had an initial spike response followed by a period of  $\text{Ca}^{2+}$  oscillations (9%; Fig. 2c) and 45% of cells had a prolonged response to agonist application that did not usually completely recover over the course of a 30 min experiment (Fig. 2d).

The observation of cells with prolonged  $\text{Ca}^{2+}$  elevations raised the possibility that this fraction of cells was damaged in a way that was not revealed by visual inspection (see Materials and methods). To test the overall health of the cells

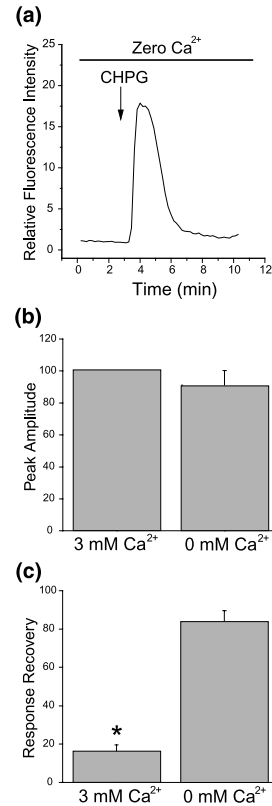


**Fig. 3** Depolarization and (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG) elicit distinct Ca<sup>2+</sup> responses. CHPG (20 s, 50  $\mu$ M)-dependent Ca<sup>2+</sup> elevations were compared with Ca<sup>2+</sup> elevations elicited by a 30 s exposure to 100 mM K<sup>+</sup>. (A, B) Data from two cells illustrate the differences in the amplitude and kinetics of Ca<sup>2+</sup> elevations in response to depolarization and CHPG application. In (a), although the peak amplitudes of the responses were similar, the CHPG response had a prolonged and oscillatory phase. In (b), the response to CHPG was  $\sim$ 30% larger than the peak Ca<sup>2+</sup> elevation elicited by depolarization and produced a prolonged Ca<sup>2+</sup> elevation. (c) Comparison of mean peak amplitudes of responses elicited by 100 mM K<sup>+</sup> and CHPG. (d) The kinetics of the two responses were quantified by measuring the fraction of recovery to baseline 5 min after CHPG (or 100 mM K<sup>+</sup>) application and were found to be significantly different ( $p < 0.05$ ,  $n = 19$ ).

and to examine their ability to buffer Ca<sup>2+</sup> elevations, we compared the responses of individual cells to depolarization with 100 mM K<sup>+</sup> external solution and to the application of CHPG. All cells examined produced Ca<sup>2+</sup> elevations in response to 100 mM K<sup>+</sup>, indicating that the cells were intact. To compare the peak amplitudes of the two types of response, the data from each cell were normalized to the peak amplitudes of the K<sup>+</sup> response for that cell. On average, CHPG responses were 70% ( $n = 19$ ) larger than those evoked by depolarization (Figs 3b and c, and see Materials and methods). The time courses of recovery of the Ca<sup>2+</sup> elevations were also significantly longer when CHPG was the stimulus, as compared with those elicited by depolarization ( $p < 0.0001$ , Fig. 3d). Furthermore, in cells that gave approximately equal amplitude Ca<sup>2+</sup> elevations, differences in the waveform and time course of the responses were still evident (Fig. 3a), indicating that distinct Ca<sup>2+</sup> mobilization pathways are activated by the two stimuli.

#### Activation of mGluR5 mobilizes Ca<sup>2+</sup> from both extracellular and intracellular sources

It has been shown in other systems that Ca<sup>2+</sup> elevations produced by activation of group I mGluRs can be triggered



**Fig. 4** In the absence of external Ca<sup>2+</sup>, (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG) stimulates a transient intracellular Ca<sup>2+</sup> elevation. Nominally Ca<sup>2+</sup>-free external solution replaced normal external solution about 1 min prior to stimulation. In the absence of external Ca<sup>2+</sup> 100% of amacrine cells tested ( $n = 59$ ) produced transient Ca<sup>2+</sup> elevation when exposed to 50  $\mu$ M CHPG (20 s). (a) Data from a representative cell shows this typically transient response. (b and c) The response amplitudes in normal and 0 mM Ca<sup>2+</sup> external solution were compared in 10 cells. The mean response amplitudes in 0 and 3 mM Ca<sup>2+</sup> were not significantly different. However, the mean duration of the responses did differ significantly ( $p < 0.05$ ). A duration analysis was performed as previously described for 100 mM K<sup>+</sup> experiments with the exception that the duration was measured as the fraction of recovery achieved within 3 min of agonist application.

by IP<sub>3</sub>-dependent mobilization of Ca<sup>2+</sup> from internal stores (Conn and Pin 1997). Alternatively, generation of cAMP could elicit Ca<sup>2+</sup> influx via cyclic nucleotide-gated channels. To examine the source(s) of the Ca<sup>2+</sup> elevation produced by mGluR5 activation in amacrine cells, CHPG was applied to cells in the absence of external Ca<sup>2+</sup>. CHPG application to amacrine cells in Ca<sup>2+</sup>-free external solution produced a Ca<sup>2+</sup> elevation in all cells examined ( $n = 59$ , Fig. 4a). The responses in Ca<sup>2+</sup>-free external solution were always transient, and typically lasted from 2 to 4 min. To confirm that the faster recovery of the Ca<sup>2+</sup> responses was due to the absence of external Ca<sup>2+</sup>, the cells were first exposed to CHPG in 0 mM Ca<sup>2+</sup>, then after recovery, the same cell was tested with CHPG in the presence of normal (3 mM) Ca<sup>2+</sup>

external solution. Mean peak amplitudes of responses elicited in normal  $\text{Ca}^{2+}$  were not significantly different from those elicited in 0 mM  $\text{Ca}^{2+}$  external solution (Fig. 4b). However, CHPG responses elicited in normal external  $\text{Ca}^{2+}$  showed significantly slower recovery than those in 0 mM  $\text{Ca}^{2+}$  ( $p < 0.05$ ,  $n = 10$ , Fig. 4c). These results indicate that the  $\text{Ca}^{2+}$  contributing to the initial peak of the response is primarily derived from internal stores. Furthermore, because the prolonged and variable part of responses elicited in normal external  $\text{Ca}^{2+}$  was never observed in 0 mM  $\text{Ca}^{2+}$ , we conclude that this component must be derived from  $\text{Ca}^{2+}$  entering the cell across the plasma membrane.

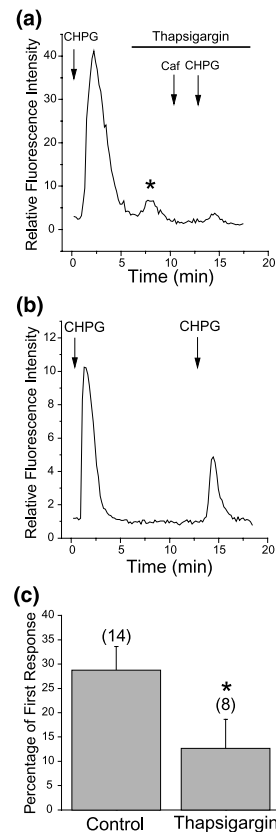
#### $\text{Ca}^{2+}$ is released from a thapsigargin-sensitive store

To establish that  $\text{Ca}^{2+}$  was being released from the endoplasmic reticulum, thapsigargin was used to disrupt sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$  pump activity (Thastrup *et al.* 1990; Inesi and Sagara 1992) and to deplete this store of  $\text{Ca}^{2+}$ . These experiments were performed in 0 mM  $\text{Ca}^{2+}$  external solution to isolate the store component and to allow us to evoke multiple, relatively brief, CHPG-dependent  $\text{Ca}^{2+}$  elevations in individual cells. Thapsigargin itself produced a small  $\text{Ca}^{2+}$  elevation in most cells that usually took about 5 min to return to baseline (Fig. 5a). After recovery, caffeine was applied to test the emptiness of the caffeine-sensitive endoplasmic reticulum store. Only those cells that failed to respond to caffeine after thapsigargin treatment were included in the data set. Application of CHPG following thapsigargin treatment produced significantly smaller  $\text{Ca}^{2+}$  elevations ( $p < 0.05$ , Fig. 5c) and in some cases (three of eight cells), failed to produce a response altogether.

Chick amacrine cells experience a general emptying of  $\text{Ca}^{2+}$  stores with prolonged exposure to  $\text{Ca}^{2+}$ -free external solutions (Gleason and Wilson, unpublished observations). In control experiments in 0 mM  $\text{Ca}^{2+}$  external solution, a second CHPG-dependent response was found to be significantly smaller than the first CHPG response ( $p < 0.001$ ,  $n = 14$ , Fig. 5B) and this reduced response is consistent with the emptying of  $\text{Ca}^{2+}$  stores previously observed. Nevertheless, a comparison of the change in mean response amplitudes for control and thapsigargin experiments revealed that thapsigargin significantly reduced the  $\text{Ca}^{2+}$  response amplitudes over the control ( $p < 0.05$ , Fig. 5c). These results indicate that the transient  $\text{Ca}^{2+}$  elevations observed in 0 mM  $\text{Ca}^{2+}$  following mGluR5 activation come from thapsigargin-sensitive internal stores.

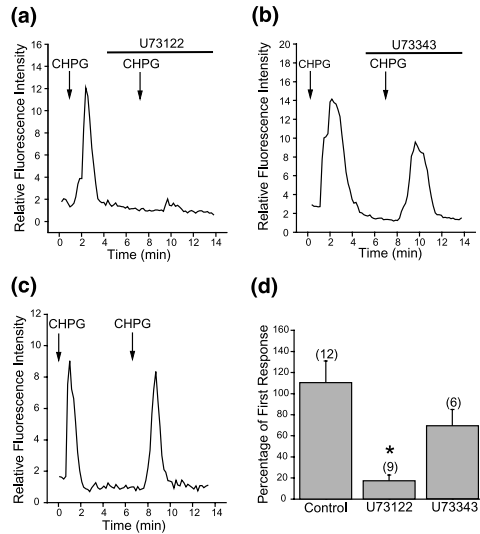
#### Phospholipase C activity is required for $\text{Ca}^{2+}$ elevations induced by mGluR5 activation

The mobilization of internal  $\text{Ca}^{2+}$  stores by group I mGluRs can be mediated by a phospholipase C-dependent pathway (Conn and Pin 1997). U73122, a specific phospholipase C antagonist (Bleasdale *et al.* 1989; Smith *et al.* 1990), was

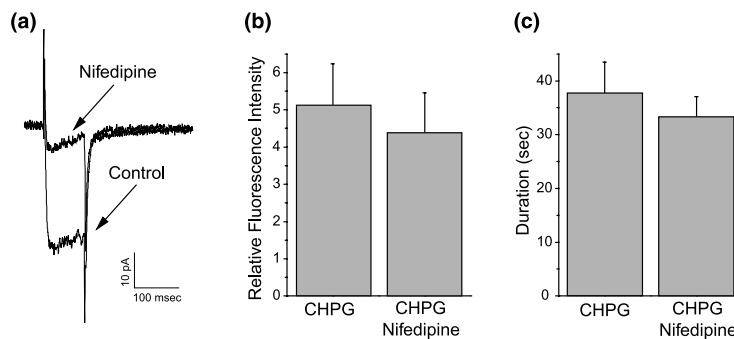


**Fig. 5** Thapsigargin-sensitive intracellular  $\text{Ca}^{2+}$  stores are involved in (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG)-dependent  $\text{Ca}^{2+}$  elevations.  $\text{Ca}^{2+}$ -free external solution replaced normal external solution about 1 min after the start of recording. (a) Treatment of cells with 100 nM thapsigargin produced  $\text{Ca}^{2+}$  elevations (asterisk) that required several minutes to return to baseline. Following the return to baseline, caffeine (20 mM) was applied to determine whether the caffeine-sensitive stores had been emptied by the thapsigargin treatment. A second application of CHPG usually produced a much smaller response. (b) Control experiments demonstrated that although the CHPG responses were reduced with prolonged exposure to 0 mM  $\text{Ca}^{2+}$ , this effect was less dramatic than specifically emptying the thapsigargin-sensitive stores. Note that the CHPG application times are the same in (a) and (b). (c) data were normalized to the peak amplitude of the first CHPG response for control and thapsigargin-treated cells. Numbers of cells are indicated in parentheses.

used to test the role of phospholipase C in the production of  $\text{Ca}^{2+}$  elevations. These experiments were also carried out in 0 mM  $\text{Ca}^{2+}$  external solution. Following the application of U73122, CHPG either produced a much smaller second response than in the controls (Fig. 6a, six of nine cells) or produced no response (three of nine cells). Experiments were also performed using U73343, a less active analog of U73122 (Fig. 6b). Although small inhibitory effects were sometimes seen in the presence of U73343, a comparison of mean amplitude changes for cells treated with U73343 and control cells (Fig. 6c) demonstrated that the responses were



**Fig. 6** Phospholipase C activity is required for (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG)-induced  $\text{Ca}^{2+}$  elevations.  $\text{Ca}^{2+}$ -free external solution was applied approximately 1 min prior to the first CHPG application. (a) Data from a representative cell showing that treatment with U73122 ( $10 \mu\text{M}$ ) almost completely abolished the second response to CHPG. (b) Treatment of a different cell with U73343 ( $10 \mu\text{M}$ ), a less active analog of U73122, produced a small decrease in the amplitude of the second response. (c) Data from a third cell shows that in the presence of  $0 \text{ mM}$   $\text{Ca}^{2+}$ , CHPG induced a similar second response approximately 6 min following recovery of the first response, demonstrating that in this time frame, the second response was not significantly affected by the absence of external  $\text{Ca}^{2+}$ . (d) A comparison of changes in mean response amplitude for first and second CHPG applications in control and in U73122 demonstrates that responses in the presence of the inhibitor were significantly reduced in amplitude. Responses from U73343-treated cells were not significantly different from control cells. Numbers of cells are indicated in parentheses. Data were normalized to the peak amplitude of the first CHPG response.



**Fig. 7** L-type voltage-gated  $\text{Ca}^{2+}$  channels do not contribute to (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG)-dependent  $\text{Ca}^{2+}$  elevations. (a) Whole cell voltage clamp recording from a representative amacrine cell.  $\text{Cs}^+$  and tetraethylammonium were included in the recording solutions to block voltage-gated  $\text{K}^+$  channels and tetrodotoxin was included to block voltage-gated  $\text{Na}^+$  channels. The cells were held at  $-70 \text{ mV}$  and stepped to a series of voltages in  $10 \text{ mV}$  increments for  $100 \text{ ms}$ . Shown is the step to  $-20 \text{ mV}$ . Bath application of  $10 \mu\text{M}$

not significantly different ( $p > 0.05$ ,  $n = 6$ , Fig. 6d). In control experiments, two CHPG responses were elicited 6 min apart, the same time frame as in experiments involving U73122. On this time frame it was possible to elicit equivalent CHPG responses (Fig. 6c,  $n = 12$ ) and the mean amplitudes for control cells and U73122-treated cells were significantly different ( $p < 0.001$ , Fig. 6d). These results demonstrate that  $\text{Ca}^{2+}$  elevations are initially dependent upon the activation of phospholipase C.

#### Pathways for $\text{Ca}^{2+}$ influx across the plasma membrane

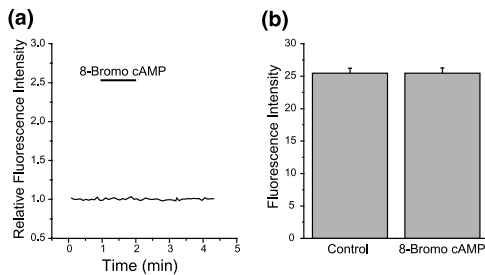
The results described above indicate that the initial source of  $\text{Ca}^{2+}$  for mGluR5-dependent  $\text{Ca}^{2+}$  elevations is internal stores. However, our comparison of response durations in normal and  $0 \text{ mM}$  external  $\text{Ca}^{2+}$  (Fig. 4c) provide evidence that after the initial release from internal stores, the  $\text{Ca}^{2+}$  elevations are prolonged by  $\text{Ca}^{2+}$  entering the cytosol across the plasma membrane. There are several possible pathways. One possibility is that activation of mGluR5 somehow leads to the opening of voltage-gated  $\text{Ca}^{2+}$  channels. We think this is unlikely, however, because in current clamp experiments, activation of mGluR5 does not lead to any obvious depolarizations (Gleason, unpublished observations). Nonetheless, to rule out this possibility, we looked at the effects of nifedipine, an L-type  $\text{Ca}^{2+}$  channel blocker. It has been previously shown that these GABAergic amacrine cells express only high voltage-activated  $\text{Ca}^{2+}$  currents. Furthermore, these currents are dihydropyridine sensitive (L-type) but are insensitive to  $\omega$ -conotoxin GVIA (N-type) and  $\omega$ -agatoxin IVA (P/Q-type) (Gleason *et al.* 1994). Thus, we conclude that these cells express L-type  $\text{Ca}^{2+}$  channels almost exclusively. In perforated-patch whole cell voltage clamp experiments,  $10 \mu\text{M}$  nifedipine inhibited the  $\text{Ca}^{2+}$  current amplitude by  $77\% (\pm 0.06, n = 5)$ ; Fig. 7a). In  $\text{Ca}^{2+}$  imaging

nifedipine blocked the  $\text{Ca}^{2+}$  current in this cell by about 80%. (b and c) Mean amplitude and duration data from  $\text{Ca}^{2+}$  measurements on fluo-3-loaded amacrine cells. Durations were measured as the width of the  $\text{Ca}^{2+}$  transient at one-half amplitude. Neither the amplitudes nor the durations of the responses of CHPG were significantly different ( $p = 0.5$  and  $p = 0.3$ , respectively,  $n = 8$ ) in the presence of  $10 \mu\text{M}$  nifedipine.

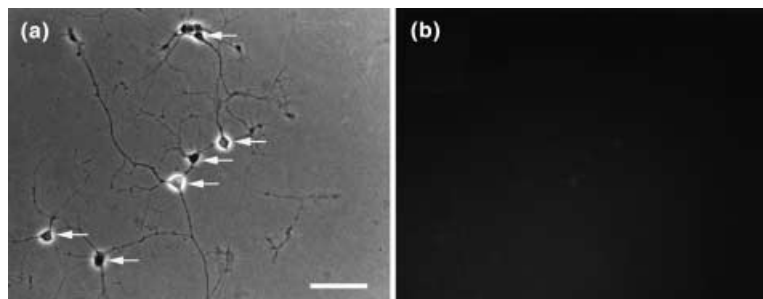


experiments, nifedipine had no significant effect on the amplitudes ( $p = 0.5$ ,  $n = 8$ , Fig. 7b) or the durations ( $p = 0.3$ ,  $n = 8$ , Fig. 7c) of the CHPG responses. These results indicate that L-type voltage-gated  $\text{Ca}^{2+}$  channels are not the pathway for CHPG-dependent  $\text{Ca}^{2+}$  influx.

Because these receptors may have the potential to couple to  $G_s$  and activate adenylate cyclase, a second possible source of  $\text{Ca}^{2+}$  influx could be via cAMP-gated channels in the plasma membrane. To determine whether amacrine cells express this sort of channel, we exposed fluo-3-loaded cells to 8-bromo-cAMP (1 mM), a cell-permeant cAMP analog capable of activating cyclic nucleotide-gated channels. If amacrine cells express these channels, and they are  $\text{Ca}^{2+}$ -permeant, we would expect to see an elevation in cytosolic  $\text{Ca}^{2+}$  when the analog binds the channels. In 16 cells tested, 8-bromo-cAMP had no effect on cytosolic  $\text{Ca}^{2+}$  levels, indicating that cyclic nucleotide-gated channels are not likely to be the pathway for CHPG-dependent  $\text{Ca}^{2+}$  influx (Fig. 8).



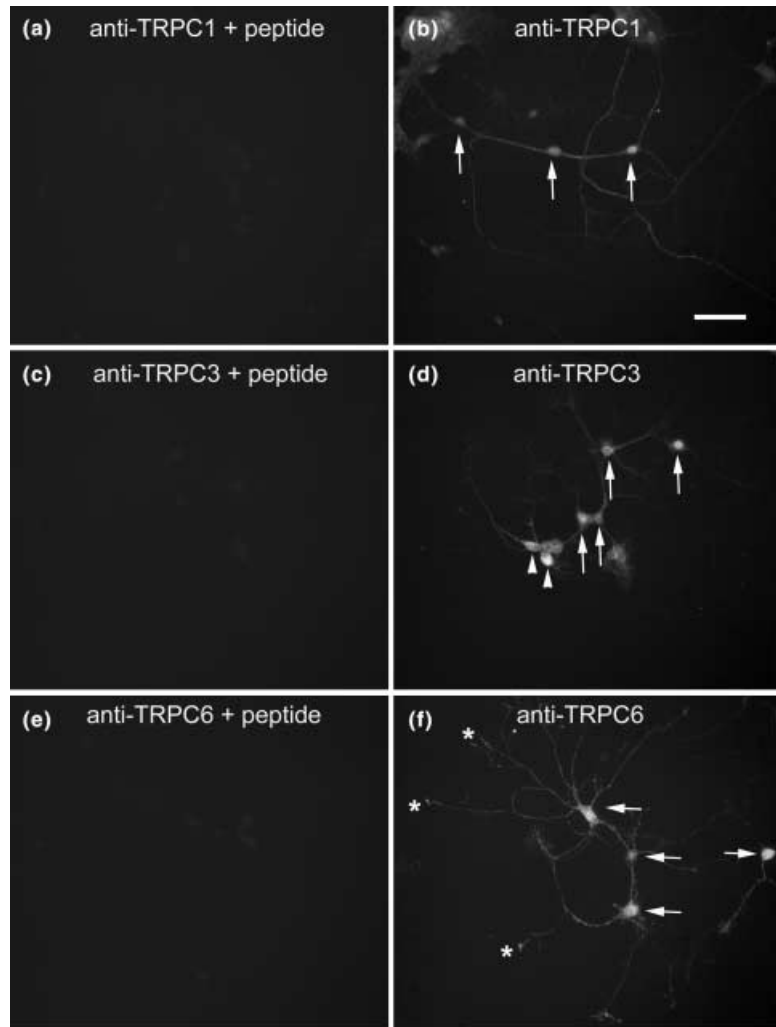
**Fig. 8** GABAergic amacrine cells do not express  $\text{Ca}^{2+}$ -permeant cAMP-gated channels. (a)  $\text{Ca}^{2+}$  measurements from a representative fluo-3-loaded amacrine cell. At the bar the external solution was exchanged for one containing 1 mM 8-bromo-cAMP. No change in relative fluorescence intensity was detected in this cell. (b) Data from 16 cells were averaged and demonstrate that no  $\text{Ca}^{2+}$  elevations were elicited by elevating cytosolic cAMP ( $p = 0.9$ ). Control data values consisted of the mean value for the three data points collected just prior to addition of 8-bromo-cAMP and the 8-bromo-cAMP data points were the average of the three data points collected just before removal of the reagent.



**Fig. 9** Secondary-only controls. (a) Phase image of the cells shown with fluorescent optics in (b). The six cells indicated by arrows can be identified as amacrine cells based on their morphology. (b) The cells in this field had been treated with the cy3-conjugated goat anti-rabbit

A third possibility is that activation of mGluR5 activates plasma membrane TRP channels. There are several potential mechanisms for activation of this class of plasma membrane channel, including store emptying, activation of phospholipase C and generation of diacylglycerol. Unfortunately, the known blockers for this family of channels (lanthanides and SKF96365, Halaszovich *et al.* 2000; 2-aminodiethyldiphenyl borate, Clapham *et al.* 2001) are extremely non-specific, making the physiological identification and study of native TRP channels problematic. In an alternative approach, we used immunocytochemistry to determine whether TRP channel subunits may be expressed by cultured GABAergic amacrine cells. We examined the binding of three polyclonal antibodies raised against peptide fragments of three different TRP channel subunits cloned from the human (TRPC1) and the mouse (TRPC3, 6). There is evidence that each of these subunits (as well as others) can be expressed in the brain (Boulay *et al.* 1997; Preuss *et al.* 1997). Both secondary antibody-only controls (Fig. 9) and antigen peptide-blocking controls (Figs 10a, c and e) revealed little, if any, non-specific binding of secondary or primary antibodies, respectively. Our examination of the binding of each of the TRP subunit antibodies on cultured amacrine cells revealed TRP subunit-like immunoreactivity. Although the same concentrations of primary and secondary antibodies were used for each, some differences in the labeling pattern and intensity were apparent among the three TRP channel antibodies. Labeling with the TRPC1 antibody gave the lowest fluorescence signal. Some amacrine cell processes were labeled (Fig. 10b) but for other amacrine cells, process labeling was not observed (not shown). The anti-TRPC3 labeling was easily discernable on both amacrine cell bodies and processes (Fig. 10d). Although we were mainly interested in amacrine cell labeling, we also observed that with the TRPC3 antibody, cone cell bodies were always the most intensely labeled cells in a field. For anti-TRPC6, labeling was also found on the cell bodies and processes of amacrine cells. Interestingly, for anti-TRPC6, process labeling often included a distinctive fluorescence signal at the distal ends

secondary antibody (1 : 1000) but no primary antibody. These results indicate a very low level, if any, of non-specific binding of the secondary antibody. Scale bar is 50  $\mu\text{m}$ .



**Fig. 10** GABAergic amacrine cells show TRPC1-, TRPC3-, and TRPC6-like immunoreactivity. (a, c and e) Three different fields of cells treated with anti-TRPC1, 3, and 6, respectively, pre-incubated with their respective antigen peptides. The three fields shown each contain a minimum of eight cells. (b) Antibody labeling with anti-TRPC1. The arrows indicate amacrine cells. Scale bar is 50  $\mu\text{m}$ . (d) Labeling of cells with the TRPC3 antibody. The arrows indicate amacrine cell bodies. The arrowheads indicate cone photoreceptors. (f) A field of cells labeled with the anti-TRPC6 antibody. The arrows indicate cell bodies of amacrine cells. The asterisks indicate labeling at process endings.

of processes (Fig. 10f, asterisks), suggesting that these channels may play a role in outgrowth or synapse formation. These results show that cultured GABAergic amacrine cells express TRP channel-like immunoreactivity which supports the possibility that activation of mGluR5 releases  $\text{Ca}^{2+}$  from internal stores and activates a TRP channel-mediated  $\text{Ca}^{2+}$  influx pathway.

## Discussion

Fundamental to understanding the consequences of mGluR activation is an understanding of the signaling pathways to which they couple. Our analysis of a partial sequence from mGluR5 expressed in the chicken retina reveals complete conservation of the amino acids previously demonstrated (Francesconi and Duvoisin 1998) to be required for coupling to  $\text{G}_q$  and  $\text{G}_s$  in rat mGluR1. It is likely that other regions of mGluR1 that may be distinctive from mGluR5 contribute to G protein coupling. Nevertheless, the complete conservation of these sequences in intracellular loops 2 and 3 is consistent

with coupling of mGluR5 to  $\text{G}_s$  as well as  $\text{G}_q$ . In support of this, heterologously expressed rat mGluR5 has been shown to stimulate cAMP production (Joly *et al.* 1995).

In chick amacrine cells, at least with respect to  $\text{Ca}^{2+}$  signaling, our data only reveal coupling to the  $\text{IP}_3$  pathway. Three pieces of evidence indicate that CHPG stimulates the  $\text{IP}_3$  pathway in amacrine cells. First,  $\text{Ca}^{2+}$  elevations were observed in the absence of extracellular  $\text{Ca}^{2+}$ , consistent with  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release from internal stores. Second, the demonstration that emptying internal  $\text{Ca}^{2+}$  stores with thapsigargin inhibits the CHPG-mediated response confirms that stores, most likely within the endoplasmic reticulum, are involved. Finally, inhibition of phospholipase C, the key enzyme in the  $\text{IP}_3$  pathway, reduces or abolishes the CHPG-dependent  $\text{Ca}^{2+}$  elevations. The absence of involvement of the cAMP pathway in  $\text{Ca}^{2+}$  signaling does not necessarily indicate its absence in amacrine cells. Clearly, activation of protein kinase A has the potential to alter the function of the cell in ways we are unable to measure with the methods reported here. The role of the cAMP pathway, if any, may be

revealed after the targets of mGluR5 activation have been identified in amacrine cells.

Our recordings of CHPG-dependent  $\text{Ca}^{2+}$  elevations reveal that the time course of the responses can vary from cell to cell. Diversity in the temporal properties of  $\text{Ca}^{2+}$  elevations has been observed within numerous cell types [for a review see Toescu (1995)]. In the amacrine cells examined here, a subset of the cells showed oscillations in cytosolic  $\text{Ca}^{2+}$  in response to mGluR5. In a heterologous expression system, activation of rat mGluR5, but not mGluR1, engendered  $\text{Ca}^{2+}$  oscillations. It was determined that this was due to phosphorylation of a specific threonine on mGluR5 by protein kinase C (PKC) (Kawabata *et al.* 1996). It is known that mGluR1 can exhibit basal activity (potentially leading to activation of PKC) in the absence of agonist (Prezeau *et al.* 1996). Additionally, there is evidence that constitutively active mGluR1 and agonist-stimulated mGluR5 can interact in cultured amacrine cells (Kreimborg *et al.* 2001). It may be that different levels of basal mGluR1 activity in different amacrine cells could underlie some of the temporal diversity in the mGluR5-dependent  $\text{Ca}^{2+}$  elevations observed.

From our experiments in zero  $\text{Ca}^{2+}$ , we know that the properties of the  $\text{Ca}^{2+}$  influx pathway play a role in determining the time course of the response. We have ruled out both L-type voltage-gated  $\text{Ca}^{2+}$  channels and cAMP-gated channels as the route of  $\text{Ca}^{2+}$  entry. However, our immunocytochemistry experiments suggest that cultured amacrine cells may express at least three TRP channel subunits. Because virtually all of the amacrine cells labeled to some extent with each of the antibodies, amacrine cells express either multiple types of homomeric TRP channel and/or an assortment of heteromeric channels. At present, TRP channels have largely been characterized as homo-multimers in heterologous expression systems. It is becoming clear, however, that the subunit content of the channels is a critical factor in determining their functional characteristics (Lintschinger *et al.* 2000; Strübing *et al.* 2001). Understanding the stoichiometry, regulation and function of TRP channels may be the key to understanding the temporal diversity of mGluR5-dependent  $\text{Ca}^{2+}$  elevations in amacrine cells.

Another aspect of amacrine cell function that has been shown to vary considerably from cell to cell is the relative contribution of different  $\text{Ca}^{2+}$  buffering mechanisms. For example, it has been shown immunocytochemically that the level of expression of the  $\text{Ca}^{2+}$  binding protein calretinin can vary among amacrine cells in culture (Carruth and Gleason 1997). Additionally, the fraction of the  $\text{Ca}^{2+}$  load through voltage-gated  $\text{Ca}^{2+}$  channels that is removed by the plasma membrane Na–Ca exchanger can range from as little as 25% to nearly 100% (Gleason *et al.* 1995). Finally, mitochondria also play a variable role in helping to buffer similar  $\text{Ca}^{2+}$  loads in these cells (Medler and Gleason 2002). Pituitary gonadotrophs normally produce vigorous  $\text{Ca}^{2+}$  oscillations. However, when mitochondrial  $\text{Ca}^{2+}$  uptake is inhibited, these

$\text{Ca}^{2+}$  oscillations are suppressed or blocked (Kaftan *et al.* 2000). This observation suggests that differences in the number and/or distribution of mitochondria could influence the temporal characteristics of  $\text{Ca}^{2+}$  elevations. Thus, variability in the  $\text{Ca}^{2+}$  buffering mechanisms in these cells may also contribute to some of the diversity observed in the mGluR5-dependent  $\text{Ca}^{2+}$  response waveform.

The ability of mGluR5 to couple to activation of the  $\text{IP}_3$  pathway has the potential to generate a variety of cellular responses. Activation of  $\text{Ca}^{2+}$ -dependent enzymes such as PKC and  $\text{Ca}^{2+}$ /calmodulin-dependent kinases have the potential to alter ion channel function (Akopian and Witkovsky 1996; Boxall 2000), voltage response properties (Goodwin *et al.* 1996) and synaptic function (Poncer *et al.* 1995; Bushell *et al.* 1999; Vetter *et al.* 1999; Gomperts *et al.* 2000). Further studies elucidating the targets of mGluR5 signaling will provide additional insights into the role of this receptor in visual processing in the inner retina.

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