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Altered G protein coupling in an mGluR6 point mutant associated with congenital stationary night blindness

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G protein coupling in a disease causing mGluR6 point mutant.

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Abbreviations:

BBS = Bungarotoxin Binding Site

BTX = Bungarotoxin

CSNB = Congenital Stationary Night Blindness

mGluR6 = Metabotropic Glutamate Receptor Type 6

SCG = Superior Cervical Ganglion

ABSTRACT

The highly specialized metabotropic glutamate receptor type 6 (mGluR6) is post-synaptically localized and expressed only in the dendrites of ON bipolar cells. Upon activation of mGluR6 by glutamate released from photoreceptors, a non-selective cation channel is inhibited causing these cells to hyperpolarize. Mutations in this gene have been implicated in the development of congenital stationary night blindness type 1 (CSNB1). We investigated 5 known mGluR6 point mutants that lead to CSNB1 to determine the molecular mechanism of each phenotype. In agreement with other studies, 4 mutants demonstrated trafficking impairment. However, mGluR6 E775K (E781K in humans) suggested no trafficking or signaling deficiencies measured by our initial assays. Most importantly, our results indicate a switch in G-Protein coupling, in which E775K loses G_o coupling, but retains coupling to G_i, which may explain the phenotype.

INTRODUCTION

The metabotropic glutamate receptors (mGluRs) are classified into three subfamilies based on their molecular identity, pharmacology, and G-Protein coupling profile (Schoepp, 2001). Group III mGluRs (4, 6, 7, 8) couple exclusively to $G_{i/o}$ proteins and negatively regulate Adenylyl Cyclase (AC) (Prezeau et al., 1994). MGluR6 is a highly specialized G-protein coupled receptor (GPCR) that is exclusively expressed in the post-synaptic dendritic region of retinal ON bipolar cells (Nakajima et al., 1993; Quraishi et al., 2007; Vardi et al., 2000). Upon activation, mGluR6 initiates a signaling cascade that ultimately results in the inhibition of a non-selective cation channel, leading to hyperpolarization of the ON bipolar cells (Masland, 2001; Nawy, 1999). Recent studies suggest that the molecular identity of the regulated cation channel is likely TRPM1 (Bellone et al., 2008; Nakajima et al., 2009; Shen et al., 2009), and a few reports suggest that regulation is mediated by $G\alpha_o$, rather than the $G\alpha_i$ subunits or by $G\beta\gamma$ (Dhingra et al., 2004; Dhingra et al., 2000; Nawy, 1999). Our lab and others have demonstrated strong coupling of mGluR6 to $G\alpha_o$ in reconstitution experiments (Tian and Kammermeier, 2006), and antibodies directed toward $G\alpha_o$ were able to block the effect of glutamate on the channel in retinal slice recordings (Nawy, 1999).

Genetic defects in the GRM6 gene (which encodes mGluR6) can lead to congenital stationary night blindness (CSNB), characterized by myopia and mild to severe impairment of night vision. In humans, mGluR6 signaling abnormalities, caused by autosomal recessive mutations, lead to complete loss of night vision, CSNB1 (Dryja et al., 2005; Zeitz et al., 2007; Zeitz et al., 2005), along with the inability to adapt to light-dark

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or cycles and reduced responsiveness to light stimuli in animal models (Pinto et al., 2007; Takao et al., 2000). CSNB2, which is characterized by less severe phenotypes, resolves from mutations occurring elsewhere in the ON visual pathway (Chang et al., 2006; Zeitz et al., 2006). Genetic studies in humans with CSNB1 and deletion of the GMR6 gene in mouse models result in the absence of the electroretinogram (ERG) b-wave (Dhingra et al., 2002; Dhingra et al., 2000; Dryja et al., 2005; Pinto et al., 2007; Zeitz et al., 2005), which reflects the depolarization of ON bipolar cells after exposure of photoreceptors to light. Interestingly, the absence of the b-wave was also observed in mice lacking $G\alpha_o$ in bipolar cells (Dhingra et al., 2002; Dhingra et al., 2000), which correlates well with the hypothesis that mGluR6 strongly couples to $G\alpha_o$ to facilitate the modulation of the cation channel.

To date, there are at least five mGluR6 point mutations that lead to CSNB in humans (P46L, G58R, G150S, C522Y, and E781K). Recent work suggests that each of these mutants exhibit impaired plasma membrane expression in HEK293 cells (Zeitz et al., 2005). Based on the location of these mutations, we anticipated that these mutants would have impaired glutamate responses due to decreased affinity for glutamate or compromised G-protein coupling. Our results confirm that four of these molecules fail to express on the plasma membrane in neurons. The mGluR6 E775K (E781K in humans) expressed and functioned normally in our preliminary assays. However, calcium mobilization data suggest that E775K couples predominantly to $G\alpha_i$, rather than $G\alpha_o$, which may explain the CSNB phenotype in humans.

MATERIALS and METHODS

SCG neuron isolation, cDNA injection and molecular methods. Detailed descriptions of the isolation and injection procedures have been previously described (Ikeda, 1997). Briefly, the SCG were dissected from adult rats and incubated in Earle's balanced salt solution (Life Technologies, Rochelle, MD) containing 0.6 mg/ml trypsin (Worthington, Freehold, NJ), 0.8 mg/ml collagenase D (Boehringer Mannheim, Indianapolis, IN) for 1 hour at 35º C. Cells were centrifuged twice, transferred to minimum essential medium (Fisher Scientific, Pittsburgh, PA), plated and placed in an incubator at 37º C until cDNA injection. Injection of cDNA was performed with an Eppendorf 5247 microinjector and Injectman NI2 micromanipulator (Madison, WI) 4-6 hours following cell isolation. Plasmids were stored at –20 ºC as a 1 µg/µl stock solution in TE buffer (10 mM TRIS, 1 mM EDTA, pH 8). MGluR6 insert was subcloned into pCDNA3.1- (InVitrogen). All receptor cDNAs were injected at 0.1 µg/µl. Neurons were co-injected with "enhanced" green fluorescent protein cDNA (0.005 µg/µl; pEGFPN1; Clontech Laboratories, Palo Alto, CA, USA) or other fluorescent marker if necessary for identification of injected cells. Point mutants (P40L, G52R, G144S, C516Y, and E775K) were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, cat# 200518) and sequence verified. BBS-mGluR6 was constructed by sub-cloning the bungarotoxin binding site sequence (TGGAGATACTACGAGAGCTCCCTGGAGCCCTACCCTGAC) between 22nd-23rd residues of the original mGluR6 clone using the overlapping extension PCR method. G_q chimeras were also constructed using the QuickChange strategy. Following injection, cells were incubated overnight at 37º C and experiments are performed the following day.

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Electrophysiology and data analysis. Patch clamp recordings were made using 8250 glass (Garner Glass, Claremont, CA). Pipette resistances were 1-3 MΩ yielding uncompensated series resistances of 1-5 MΩ. Series resistance compensation of 70-80% was used in all recordings. Data was recorded using an Axopatch 200B patch-clamp amplifier from Molecular Devices (Foster City, CA). Voltage protocol generation and data acquisition were performed using custom data acquisition software (generously donated by Stephen R. Ikeda, NIAAA) on a Macintosh G3 computer with an Instrutech ITC18 data acquisition board (HEKA Elektronik, Germany). Currents were sampled at 0.5-5 kHz low-pass filtered at 3-5 kHz using the 4-pole Bessel filter in the patch clamp amplifier, digitized, and stored on the computer for later analysis. All patch-clamp experiments were performed at 21-24 °C (room temperature). Data analysis was performed using Igor software (WaveMetrics, Lake Oswego, OR). For calcium current recordings in SCG, the external (bath) solution contained (in mM):145 tetraethylammonium (TEA) methanesulfonate (MS),10 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 glucose, 10 $CaCl_2$, and 300 nM tetrodotoxin, pH 7.4, osmolality 320 mOsm/kg. The internal (pipette) solution contains: 120 N-methyl-Dglucamine (NMG) MS, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 $CaCl_2$, 4 MgATP, 0.3 Na_2GTP, and 14 tris-creatine phosphate, pH 7.2, osmolality 300 mOsm/kg. Pertussis toxin (PTX) (List Biological Lab. Inc. Campbell, CA) was applied at 500 ng/ml media overnight at 37° C.

PTX-insensitive reconstitution recordings. A good stoichiometric balance between the α and βγ subunits was necessary to perform the reconstitution experiments. As previously described, the triple pulse protocol that produces the "pre" and the "post" currents is an effective way to detect an imbalance (Kammermeier et al., 2003). Excess

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of Gα subunits will result in a "pre" current that is slightly higher than the "post" current due to α-GDP serving as a "sponge" for Gβγ. By contrast, surplus of Gβγ will result in a "pre" current that is already inhibited (Herlitze et al., 1996; Ikeda, 1996; Ikeda and Dunlap, 1999). To overcome this obstacle, we only analyzed cells that had a post/pre ratio between 1-1.3. Ratios between these values are indicative a good stoichiometric G-protein subunit balance (Kammermeier et al., 2003). The α subunit plasmids were injected at 5-6 ng/µl and $G\beta_1$ and $G\gamma_2$ plasmids were injected at 8-10 ng/µl final concentration.

Confocal Microscopy. Injected SCG neurons (in glass coverslips) with the desired plasmid along with "enhanced" green fluorescent protein cDNA to confirm expression were incubated overnight at 37º C. 16-18 hours post injection cells were transferred into a perfusion chamber and washed with PBS, followed by treatment with 1µM α-bungarotoxin AlexaFluor 647 conjugate (Molecular Probes, Eugene, OR) for 1 minute. Cells were washed again with PBS, and imaging was performed using an inverted Nikon microscope through a 40X oil immersion objective lens. Confocal images were obtained by utilizing the EZ-C1 3.60 software program (Nikon, Melville, NY). Cells were excited at 488 and 633 nm and signal was detected at 530 ± 30 and 688 ± 20 nm for GFP and AlexaFluor 647 respectively.

Digital imaging of intracellular Ca^{2+} in muscarinic type 3 (m3) stable HEK293 cells. M3 HEK293 cells, obtained from Dr. Trevor Shuttleworth, (University of Rochester, Rochester, NY) were loaded with 2 mM of the Ca^{2+}-sensitive dye fura-2 AM at 37º C for 15-20 minutes. Transfected cells were loaded with Fura-2 AM then transferred into a perfusion chamber. Cells were perfused in HEPES-buffered physiological saline con-

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taining (in mM) 137 NaCl, 0.56 $MgCl_2$, 4.7 KCl, 1 Na_2HPO_4, 10 HEPES, 5.5 glucose, and

1.26 $CaCl_2$, pH 7.4. Imaging was performed using an inverted Nikon microscope

through a 40X oil immersion objective lens (numerical aperture, 1.3). Fura-2 AM loaded

cells were excited alternately with light at 340 and 380 nm by using a monochrometer-

based illumination system (TILL Photonics, Pleasanton, CA), and the emission at 510

nm was captured by using a digital frame transfer CCD camera. Images were captured

every 2 s with an exposure of 40 ms and 4 by 4 binning. Analysis was performed by

TILL Vision software. Any signal below 0.1 ratio was designated as background noise.

The receptors and the Gq chimeras were transfected at 1 μg/35 mm dish, β_1 and γ_2

plasmids were transfected at 0.5 μg/35 mm dish, and finally dSRed-nuc was transfected

at 0.2-0.3 μg/35 mm dish (for the supplementary experiment), and cherry for the Ca^{2+}

mobilization experiments.

RESULTS

**Modulation N-type ($Ca_{v2.2}$) calcium channels in superior cervical ganglion
neurons (SCGs) by mGluR6 and five CSNB point mutants.** Our initial hypothesis re-

garding the CSNB point mutants evolved from the idea that these receptors have a re-

duced or diminished ability to respond to glutamate. Thus, we examined modulation of

$Ca_{v2.2}$ channels in SCG neurons by each point mutant to examine receptor activity. The

triple pulse voltage protocol, shown in Fig. 1A (inset), was used to assess channel activ-

ity and consists of two test pulses to +10 mV from a -80 mV holding potential, separated

by a strong depolarizing step to +80 mV. The +80 mV step partially reverses the well

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characterized voltage-dependent, Gβγ mediated inhibition (Elmslie et al., 1990; Ikeda, 1991; Ikeda, 1996).

To determine the effects of five CSNB point mutations on mGluR6 signaling, the corresponding mutants were made in the rat mGluR6 sequence and each was expressed in SCG neurons. Fig.1A, shows a typical time-course of an SCG neuron expressing mGluR6, illustrating inhibition of calcium currents during glutamate application. No effect was observed in uninjected or GFP only injected cells (data not shown).

In Fig. 1B, the glutamate concentration-response curve for the wild-type and each point mutant is shown. Four of the mutants, P40L, G52R, G144S, and C516Y showed no response to glutamate. By contrast, cells expressing mGluR6 E775K showed responses similar to those of the wild-type mGluR6. We noticed a slight right shift in the EC_{50} values of mGluR6 E775K concentration-response curve (EC_{50} for mGluR6 was 8 μM and 20 μM GLU for the mutant). However, at 100 μM glutamate applications the responses were identical (45 $\pm$ 5% and 44 $\pm$ 2% current inhibition for mGluR6 and E775K respectively). These data suggest that four of the mGluR6 CSNB point mutants are non-functional. By contrast, mGluR6 E775K appears to function similarly to the wild-type.

Four CSNB point mutants lack plasma membrane expression. To determine why the non-functional mutant receptors lacked responses, the plasma membrane expression of each was examined. Zeitz et al. (2007), reported that all of the CSNB point mutants are retained in the ER when overexpressed in HEK293 cells. To evaluate plasma membrane expression in SCG neurons, wild-type mGluR6 and each CSNB mu-

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tant were tagged with a N-terminal, extracellular bungarotoxin (BTX) binding site (BBS) (Sekine-Aizawa and Huganir, 2004). The plasma membrane expression of each receptor was then examined by application of a fluorescent tagged α-bungarotoxin to SCG neurons expressing each receptor. Fig. 2 illustrates representative SCG injected neurons with the indicated plasmid along with pEGFP. Cells expressing GFP alone showed no membrane fluorescence after treatment with α-bungarotoxin-AlexaFluor 647 (shown in red). Further, no detectable membrane labeling was evident in cells expressing P40L, G52R, G144S, or C516Y. However, cells expressing the wild-type or mGluR6 E775K mutant demonstrated comparable surface membrane labeling (Fig. 2). To verify that the BTX binding site tag did not interfere with receptor function, electrophysiological experiments were performed by monitoring calcium channel modulation by 100 μM glutamate in cells expressing mGluR6 or BBS-mGluR6. Similar responses were observed in SCG neurons expressing each receptor (Fig. 2). Calcium currents were inhibited 43 $\pm$ 4% (n=14) and 48 $\pm$ 5% (n=8) in cells expressing mGluR6 and BBS-mGluR6, respectively.

E775K does not activate Gα$_o$. Although most of our labeling data correlated with the previous report (Zeitz et al., 2007), one CSNB mutant, mGluR6 E775K, was able to traffic to the plasma membrane and function similarly to the wild-type mGluR6 in SCG neurons. Because wild-type mGluR6 appears to act in the retina through Gα$_{oA}$ stimulation (Dhingra et al., 2002) it is possible that the E775K mutant may produce CSNB due to an inability to activate Gα$_{oA}$, rather than lack of expression or due to trafficking defects. Because the E775K residue resides in the third intracellular loop (i3), a change in

the G-protein coupling seemed possible (Francesconi and Duvoisin, 1998). Therefore, the G-protein coupling of mGluR6 E775K was examined more closely.

As with wild-type mGluR6, E775K responses were abolished by overnight pertussis toxin (PTX) treatment, indicating that the mutant coupled exclusively to $G_{i/o}$ proteins (see Fig. 3). Calcium currents were inhibited $45 \pm 4\%$ (n=6) by 100 µM GLU and $6 \pm 1\%$ (n=5) with overnight PTX treatment when mGluR6 was expressed in SCG neurons. Similar responses were obtained ($42 \pm 3\%$, n=11 and $3 \pm 1\%$, n=7 when mGluR6 E775K was expressed.

Next, the possibility that the E775K mutation changes from a predominantly G_o-coupled to a predominantly G_i-coupled receptor was tested. Therefore, coupling of mGluR6 E775K to specific G-proteins was examined by reconstitution using PTX-insensitive $G_{i/o}$ protein mutations of the cysteine residue, near the end of C-terminus (351/2 position, the site for ADP ribosylation) to glycine, rendering the G-proteins insensitive to PTX, as described previously (Bahia et al., 1998).

SCG neurons expressing either mGluR6 wild-type or E775K were treated with PTX to inactivate endogenously expressed $G\alpha_{i/o}$ proteins and calcium current modulation was examined when $G\beta_1$ and $G\gamma_2$ were co-expressed along with each $G\alpha_{i/o}CG$ PTX-insensitive protein (see Fig. 3).

Consistent with previous results (Tian and Kammermeier, 2006), reconstitution with $G\alpha_{oA}CG$ resulted in a strong coupling to mGluR6 wild-type ($32 \pm 1\%$ inhibition n=7). However, no coupling was detected with E775K mutant ($2 \pm 1\%$ inhibition n=6). Surprisingly, no coupling was observed with $G_{i1}CG$, $G_{i2}CG$, or $G_{i3}CG$ either (3 ± 2, 3 ± 2, and $4 \pm 2\%$ inhibition respectively) (Fig. 4), despite a strong PTX-sensitive signal when

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mGluR6 E775K was expressed alone. Thus, although the E-K mutation appeared to abolish signaling through $G\alpha_{oA}$, it is possible that the reconstitution assay is not sufficiently sensitive, or that the C-G mutation may selectively disrupt coupling to the mutant receptor. Therefore, an alternative approach to investigate mGluR6 E775K signaling was necessary.

E775K couples predominantly to G_i proteins. As an alternative strategy to investigate mGluR6 E775K signaling, Ca^{2+} mobilization was examined in m3 (muscarinic type 3) HEK293 stable cell lines expressing $G_qG_{i/o}$ chimeric proteins in which the extreme C-terminus amino acids has been exchanged to allow activation by mGluR6 (Blahos et al., 1998). Gq chimeras have been widely used to study $G_{i/o}$ coupled GPCR signaling (Kowal et al., 2003; Kowal et al., 2002; Walker et al., 2005; Zhang et al., 2003).

To this end, $G_qG_{oA(9)}$ and $G_qG_{i1/2(9)}$ chimeras were generated (the last 9 residues for G_{i1} and G_{i2} are identical), and used to perform Ca^{2+} fura-2 AM imaging experiments in m3 HEK293 stable cell lines. M3 receptors couple well to native G_q, which would serve as an internal control when 300 nM carbachol was applied. These cells, were heterologously transfected with each receptor (mGluR6 or E775K), the G_q chimera, and $G\beta_1\gamma_2$ constructs, along with cherry (a red fluorescent protein) to identify expressing cells.

Fig.5A shows representative Ca^{2+} traces illustrating Ca^{2+} signals when each agonist was applied. In cells transfected with the receptor alone no signal was detected upon glutamate application (mGluR6 151 cells analyzed; E775K 149 cells). These results suggested that neither mGluR6 nor mGluR6 E775K could induce an intracellular Ca^{2+} signal via natively expressing G-proteins. When the wild-type receptor was coex-

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pressed with $G_qG_{oA(9)}$ and $G\beta_1\gamma_2$, a strong Ca^{2+} response was detected in 31 of 208 cells analyzed. By contrast only 1 of 82 cells expressing mGluR6 E775K produced a detectable response, and this response was weak (Fig. 5 A,B). These results correlated well with the electrophysiological data (Fig. 3). When $G_qG_{i1/2(9)}$ was expressed some signaling was detected with mGluR6 (3 out of 88 cells). However, this signal was weaker and less frequent than when $G_qG_{oA(9)}$ was expressed. Interestingly, mGluR6 E775K was able to signal in response to glutamate in 15 out of 460 $G_qG_{i1/2(9)}$ expressing cells (Fig. 5A, B). These data confirm that mGluR6 couples strongly to $G\alpha_o$ and weakly to $G\alpha_i$, while demonstrating that E775K mutant receptor predominantly activates $G\alpha_i$. Further, since this mutation causes CSNB in humans, effectively resulting in loss of function, these data support the conclusion that mGluR6 in retinal ON bipolar cells produces its primary signal via $G\alpha_o$ activation, rather then $G\alpha_i$ or $G\beta\gamma$.

DISCUSSION

We report here that a disease causing mGluR6 mutant (E775K) functions and expresses similar to the wild-type receptor in SCG neurons. Further investigation of the signaling cascade revealed that mGluR6 E775K is incapable of activating $G\alpha_o$ and primarily couples to $G\alpha_i$ proteins. Overall, the data presented here suggest that mGluR6 primarily functions through $G\alpha_o$ proteins in retinal ON bipolar cells, and an inability of the receptor to activate $G\alpha_o$ results in loss of function, leading to CSNB.

Three of the CSNB mGluR6 point mutants examined here, P40L, G52R, and G144S are located in the Venus Fly-Trap Domain (VFD) near the agonist binding site (Bessis et al., 2000; Rosemond et al., 2002). The C516 residue is located in the Cys-

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teine Rich Region (CRR) of the receptor, which separates the VFD from the 7TM region and is believed to be important in the intramolecular signal transmission (Rondard et al., 2006). Therefore, the mechanism of loss of function for each seemed apparent. Surprisingly, each of these mutants lacked function not due to a predicted deficit in ligand binding or intramolecular signal transduction, but due to loss of plasma membrane expression. These results are consistent with those of Zeitz et. al. in HEK293 cells, and support the notion that mGluR6 trafficking is tightly regulated such that even small structural perturbations can severely alter receptor trafficking.

The E775 residue is located in the third intracellular loop of the receptor. The second (i2) and the third (i3) of mGluRs participate in G-protein contact and activation. Interestingly the i3 loop of all mGluRs contains a conserved region, and single point mutations in this region have been known to impair receptor/G-protein coupling (Francesconi and Duvoisin, 1998).

According to the functional data presented here, E775K produced responses similar to the wild-type receptor at 100 μM GLU with only a slight shift in potency (see Fig. 1). Membrane surface labeling indicated that the mutant receptor is present on the plasma membrane in SCGs following nuclear cDNA injection (Fig. 2), and in HEK293 cells when transfected (see supplementary Fig. 1). However, the observation that an mGluR6 point mutation that resulted in a nearly normal functioning receptor was puzzling.

Wild-type mGluR6 couples to G_o proteins in ON bipolar cells (Nawy, 1999) and in reconstitution studies in SCG neurons (Tian and Kammermeier, 2006). Further, the primary signal transduction induced by mGluR6 in retinal ON bipolar cells, the inhibition of

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a non-selective cation conductance, presumably TRPM1 (Shen et al., 2009), appears to require activation of $G\alpha_o$, as disruption of neither $G\alpha_i$ nor $G\beta\gamma$ can occlude this effect (Dhingra et al., 2002; Nawy, 1999). Therefore, a change in G-protein coupling preference of mGluR6 from $G\alpha_o$ to $G\alpha_i$ could result in CSNB even if the mutant receptor is otherwise functional. This hypothesis was tested for mGluR6 E775K with two strategies. First, by reconstitution of signaling using PTX-insensitive mutant $G\alpha$ proteins in SCG neurons, and second by examining intracellular calcium signaling with fura-2AM in HEK293 cells expressing $G\alpha_q G\alpha_{i/o}$ chimeric proteins.

Unfortunately, reconstitution of signaling with the mutant receptor in SCG neurons with PTX-insensitive G-proteins could not produce a complete answer. The data suggested a loss of E775K coupling to $G_{oA}CG$. However, when the G_iCG mutants were utilized, no coupling was observed. These negative results may have resulted from a weak expression of the G-protein mutant in SCGs, low sensitivity of the assay (channel modulation), or the possibility that mGluR6 E775K coupled weakly to more than one G_i protein, but not robustly to any one in isolation.

To this end, a more sensitive assay, Ca^{2+} imaging experiments with fura-2AM, was employed. We constructed $G_q G\alpha_{i/o}$ chimeras in which the last 9 residues were swapped with that of the G_{oA}, G_{i1}, and G_{i2} (the last 9 residues of Gi1 and Gi2 are identical, so this construct was called $G_q G_{i1/2}$). Similar constructs have been used to study mGluR2 and mGluR4 signaling in CHO cells (Kowal et al., 2003). In accordance with our electrophysiological data, mGluR6 was able to strongly couple to $G_q G_{oA}$, with less robust coupling to $G_q G_{i1/2}$. However, mGluR6 E775K failed to couple to $G_q G_{oA}$, but elicited substantial Ca^{2+} signals when expressed with $G_q G_{i1/2}$. It is important to note that during

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these experiments several plasmid constructs were required (receptor, chimera, $G\beta_1$, $G\gamma_2$, and cherry), so the probability of getting all the components in any one cell is low. Therefore, the low number of responding cells in each experiment was expected.

In conclusion, we demonstrate here an alternative explanation for the CSNB phenotype (for E781K mutant in humans) as has been previously proposed. These results indicate mGluR6 E775K functions and expresses like the wild-type receptor, but its ability to activate $G\alpha_{oA}$ has been diminished. The mutant receptor retains the capability to activate G_i proteins in heterologous and reconstitution assays, but that may not be sufficient to produce effective signaling in retinal ON bipolar cells.

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FOOTNOTES

None

LEGENDS FOR FIGURES

Fig. 1. Inhibition calcium channels by mGluR6 and CSNB point mutants. A, a representative time course and current trace (inset) of mGluR6-mediated inhibition of the native calcium currents in SCG neurons at indicated agonist (GLU) concentrations. B, concentration-response curve of mGluR6 and CSNB point mutants. The wild-type and the mutants are represented by the indicated symbol. The data is represented as average calcium current percent inhibition $\pm$ SEM for mGluR6 (n=11), P40L (n=3), G52R (n=6), G144S (n=4), C516Y (n=7), and E775K 100 μM (n=23), 30 μM (n=12), 10 μM (n=9), 1 and 0.1 μM GLU (n=4).

Fig. 2. Surface membrane labeling of mGluR6 and CSNB point mutants in SCG neurons. BBS tagged wild type and point mutants were co-injected with GFP (green) and treated with α-bungarotoxin Alexa Fluor 647 conjugate (red). Average calcium current inhibition by glutamate for the wild-type (white bar) and BBS tagged mGluR6 (black bar) in SCGs is shown in the bar graph. Number of cells is indicated in parentheses.

Fig. 3. Reconstitution of coupling to mGluR6 (white bars) and mGluR6 E775K mutant (black bars) using PTX-insensitive $G_{oA}CG$. Average calcium current inhibition ($\pm$ SEM) in SCG neurons expressing mGluR6 or E775K alone, following overnight PTX treatment, and in combination with PTX-insensitive $G_{oA}CG$ (and $G\beta_1$ and $G\gamma_2$) are shown. Number of cells is indicated in parentheses.

Fig. 4. Reconstitution of coupling to mGluR6 E775K using PTX-insensitive $G_{i1}CG$, $G_{i2}CG$, or $G_{i3}CG$. Average calcium current inhibition ($\pm$ SEM) in SCG neurons expressing E775K alone or in combination with the indicated PTX-insensitive Gα protein (plus β_1 and γ_2) are shown. Number of cells is indicated in parentheses.

Fig. 5. Reconstitution of mGluR6 and mGluR6 E775K coupling in m3 (muscarinic type 3) stable HEK293 cells. A, representative traces from cells expressing mGluR6 (left) and mGluR6 E775K (right) alone or in combination with the indicated chimera with $G\beta_1$ and $G\gamma_2$. The scale bars represent 2 ratio units and 60 sec. B, scatter plots showing the fluorescence intensity ratios (F340/F380) of mGluR6 or E775K alone or in combination with the indicated chimera (including $G\beta_1$ and $G\gamma_{2)}$. Number of analyzed cells is indicated in the text.
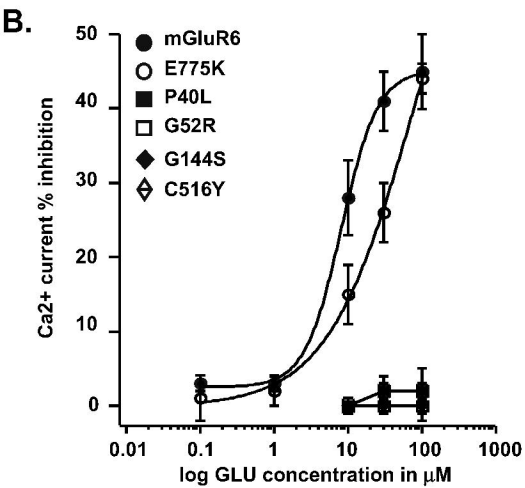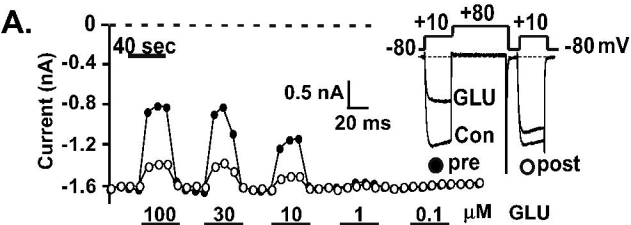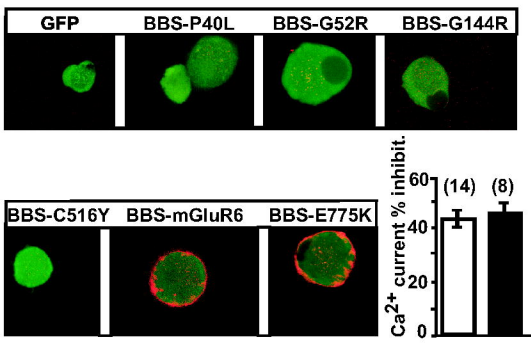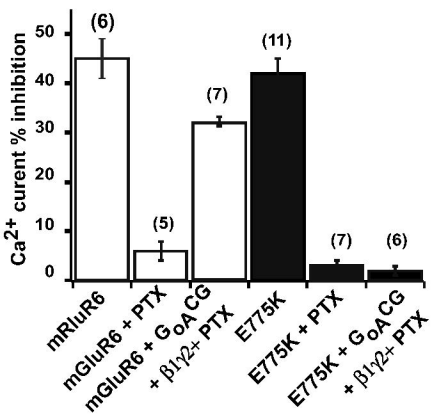
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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5