Functional Coupling of a Human Retinal Metabotropic Glutamate Receptor (hmGluR6) to Bovine Rod Transducin and Rat $G_o$ in an in Vitro Reconstitution System*

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The cDNA encoding hmGluR6, appended with a 15-amino acid antibody epitope (1D4), was transiently transfected in COS-7 cells. The receptor was purified from COS cell membranes using an antibody affinity column. The purified receptor was then reconstituted into lipid vesicles, and its ability to activate either transducin, the rod photoreceptor-specific GTP-binding protein, or the $\alpha$ subunit of $G_o$ was assayed in vitro using a guanosine 5’-3-O-(thio)triphosphate binding assay. Activation of both transducin and $G_o$ was observed. The rate of $G_o$ activation was 18-fold greater than the rate of transducin activation. This indicates that the coupling of mGluR6 to $G_o$ is more efficient and suggests that $G_o$ may be involved in coupling to mGluR6 in ON-bipolar cells.

Glutamate, the major excitatory neurotransmitter in the central nervous system, activates both ionotropic receptors and metabotropic receptors (mGluR) coupled to GTP-binding proteins (G-proteins). Recent molecular cloning studies have identified eight different subtypes of glutamate metabotropic receptors (1, 2). These receptors possess seven putative membrane-spanning domains preceded by a large extracellular domain that probably functions as the ligand-binding domain (3, 4). Despite having an overall design similar to other G-protein-coupled receptors, mGluRs do not have sequence similarity to other G-protein-coupled receptors, except the recently cloned $\gamma$-aminobutyric acid $\beta$ receptors where the identity is only 18–23% (5). The coupling of mGluRs to G-proteins and second messenger systems has been studied in heterologous expression systems using either Xenopus oocytes or stable cell lines. From these studies, it appears that a variety of different G-proteins are involved in the coupling of mGluRs to either phospholipase C or adenyl cyclase (1, 2).

In the vertebrate retina, glutamate is the neurotransmitter released by photoreceptors in the dark. At the synapse between photoreceptors and bipolar cells, glutamate functions as both an excitatory and inhibitory neurotransmitter, exciting OFF-bipolar cells by opening ionotropic glutamate receptors and inhibiting ON-bipolar cells by activating a metabotropic receptor (6, 7). L-2-Amino-4-phosphonobutyrate (L-AP4) mimics the action of L-glutamate at ON-bipolar cells and selectively hyperpolarizes these cells (8, 9). Based on a series of physiological and pharmacological studies, it has been suggested that both L-glutamate and L-AP4 activate the G-protein-coupled mGluR expressed in ON-bipolar cells and activate a cGMP biochemical cascade similar to the light-activated phototransduction cascade in the rod and cone photoreceptors. It has further been suggested that upon activation, mGluR6 can stimulate a transducin-like G-protein that activates a cGMP phosphodiesterase (10, 11). The resulting decrease in intracellular concentrations of cGMP leads to the closure of cGMP-gated, cation-selective ion channels and results in hyperpolarization of ON-bipolar cells (12–14). Light, which hyperpolarizes the photoreceptors, transiently depolarizes the ON-bipolar cells. Thus, this metabotropic receptor, mGluR6, is likely the only metabotropic receptor that directly mediates synaptic transmission.

Although there is considerable evidence to support the involvement of an mGluR (mGluR6) in mediating the dark, hyperpolarizing response of ON-bipolar cells, there is little evidence supporting the idea that the components of the mGluR6-linked second messenger pathway are similar to the proteins involved in the cGMP cascade found in photoreceptors. Using genetically engineered mice, it has recently been demonstrated that mGluR6 mediates the synaptic effects of glutamate in both rod and cone ON-bipolar cells and is essential for normal visual processing (15, 16). MGLuR6 was originally cloned from a rat retinal cDNA library and expressed in Chinese hamster ovary cells (17). In this heterologous expression system the activation of MGLuR6 inhibited forskolin-stimulated adenyl cyclase with no indication of involvement in a cGMP cascade (17). A similar observation has been made with the human homolog, hMGLuR6 (18). An alternate approach to attempt to link mGluR6 to a cGMP biochemical cascade has been to probe bipolar cells with immunological probes. No cross-reactivity was observed in vertebrate bipolar cells with antibodies to transducin, cGMP phosphodiesterase, cGMP-gated channel, or arrestin, which are some of the proteins involved in mediating the cGMP cascade of rod photoreceptors, suggesting that the proteins involved in the second messenger system in bipolar cells are not identical to those expressed in rod photoreceptors (19, 20). A recent physiological study demonstrated that the cGMP-gated channel expressed in cat bipolar cells has properties different from the cyclic nucleotide-gated channels found in either photoreceptors or olfactory receptors (14), suggesting that a different class of cGMP-gated channels may be expressed in ON-bipolar cells. Furthermore, Noga et al. found that a $G_o$-specific antibody cross-reacted with rod bipolar cells, suggesting that mGluR6...
may activate a Go-like G-protein (19). Additional experiments are needed to identify the second messenger system that links mGluR6 activation to hyperpolarization of retinal ON-bipolar cells.

To address the coupling of mGluR6 to a G-protein, we examined it in an in vitro reconstitution system. We cloned and modified the human mGluR6, expressed it in COS-7 cells, purified it, and assayed its function in an in vitro GTP binding assay. Our results demonstrate that mGluR6 can activate both transducin and Go in an agonist-dependent fashion. The rate of Go activation was 18-fold greater than the rate of transducin activation, suggesting that a Go type G-protein may be activated by mGluR6 in vivo.

EXPERIMENTAL PROCEDURES

Materials—All reagents, except where indicated, were purchased from Sigma. t-AP4 was purchased from Tocris Neuramin. [35S]GTPγS was from NEN, and nonradioiodlated GTPγS (tetra lithium salt) was from Boehringer Mannheim. LipofectAMINE was from Life Technologies, Inc. Gt10 was obtained from Calbiochem. Frozen bovine retinas were obtained from Schenk Packing Company (Stanwood, WA). The monoclonal antibody rhodopsin 1D4, which is specific for the C terminus of rhodopsin has been previously described (21). Peptide I (DEASTTVSK-
AEXPTA) was purchased from the American Peptide Co., Inc.

cDNA Cloning and Modification of the hmGluR6 Clone—Two million recombinants of human adult hippocampal A2AP1 cDNA library (Stratagene, San Diego, CA) using oligo(dT) and randomly primed poly(A)+ RNA, were screened with a C-terminal (nt 1851–2750) rat mGluR4 probe (22). The mGluR4 probe was generated by random priming the gel purified fragment using [32P]dCTP (Random Primed DNA Labelling Kit, Boehringer Mannheim). Hybridizations were carried out overnight at 60 °C in 5 × SSC/5 × Denhardt’s/0.2% SDS/50 mg/ml herring testis DNA. Washes were done for 30 min each at 65 °C in 2 × SSC/0.2% SDS followed by two 1 × SSC/0.2% SDS washes. One plaque hybridized to the C-terminal fragment of mGluR4 and was rescued into Bluescript SK(−) phagemid by in vivo excision and characterized. One partial mGluR6 cDNA (pBS-cmR1; nt 1463–2123) was obtained. Unless otherwise noted, the nucleotide numbers in the text refer to the human mGluR6 coding and 3′-untranslated sequences reported here, with negative numbers representing 5′-untranslated sequence. One million recombinants from a gtl0 human retinal cDNA library (CLONTECH, Palo Alto, CA) were hybridized with a 32P-labeled 610-base pair PstI fragment isolated from pBS-cmr1 (nt 1541–2123) at 30% formamide, 5 × sodium chloride/sodium phosphate/EDTA buffer (SSPE), 5 × Denhardt’s solution, 4% SDS, and 2 mg/ml boiled herring sperm at 42 °C. The filters were washed at 0.2 × SSPE, 0.2% SDS; 65 °C.

Twelve partial hmGluR6 cDNA clones were obtained (METAB65–METAB76) from this screen. Sequence analysis of these cDNAs revealed that METAB75 was the largest partial mGluR6 cDNA (nt 794–3105) isolated. To obtain the 5′ portion of human mGluR6, a specifically primed human retinal gtl0 cDNA library was constructed using an antisense mGluR6 oligonucleotide (nt 1083–1058) to prime human retinal poly(A)+ RNA essentially as described by Gubler and Hoffman (23) and Lapeyre and Amalric (24). Approximately 1.3 × 106 recombinants from the gtl0 specifically primed library were screened with a 582-base pair Smal fragment (nt 820–1401) from METAB75 at the hybridization and washing stringencies described above for the retinal library screening. Twenty hybridizing plaques were identified in this screen and eight putative human mGluR6 clones (METAB77 to METAB85) were isolated and characterized. The full-length pCMV−2(−SA/SD)hmGluR6 construct was prepared using the METAB85 (nt −84 to 1083) and METAB75 (nt 794–3105) cDNAs. Initially, 1- and 2.3-kilobase EcoRI fragments isolated from METAB85 and METAB75, respectively, were subcloned into the pGEM7Z vector (Promega, Madison, WI). An

![Figure 1](image1.png)

**Fig. 1.** Analysis of the purified hmGluR6 by silver stain and immunoblot. A, silver stain analysis of proteins in a COS-7 cell membrane preparation after transfection with the modified vector pm-t2 containing the modified hmGluR6 gene and subsequent purification of hmGluR6 using immunoffinity chromatography. Lane 1 contains 5 μl of solubilized membranes from a 600-μl membrane preparation prepared from six 10-cm tissue culture plates with cells at a density of 5 × 10⁶ cells/plate. Lane 2 is 20 μl of a 300-μl lipid vesicle-receptor preparation that has receptor purified from six tissue culture plates as described above. These samples were analyzed on a 15% SDS/PAGE gel. B, immunoblot analysis of purified hmGluR6 and bovine rhodopsin. Lane 1 is 20 μl of a purified receptor-vesicle preparation, and the control in lane 2 is 15 pmol of bovine rhodopsin. The proteins were initially separated on a 10% SDS/PAGE gel.

![Figure 2](image2.png)

**Fig. 2.** L-AP4 activated hmGluR6 activates bovine transducin. A, the time course for the binding of GTPγS to transducin as catalyzed by L-AP4-activated hmGluR6 purified from transfected COS cells and reconstituted into lipid vesicles. The assay was performed as described under “Experimental Procedures.” 6 μm L-AP4 (●) was used in this assay and compared with an identical reaction in the absence of agonist (○). The rate of 0.065 pmol of GTPγS bound/min is the slope of the line. This rate is approximately 4-fold greater than the rate observed in the absence of agonist. The data are from one of six similar experiments (Table I). B, light-activated bovine rhodopsin activates bovine transducin. The time course for the binding of GTPγS to transducin as catalyzed by light-activated bovine rhodopsin purified from transfected COS cells and reconstituted into lipid vesicles. The assay was performed as described under “Experimental Procedures.” Three aliquots were assayed in the dark (●) at 30-s intervals. The remaining reaction was exposed to room light for 30 s, and five additional aliquots were removed and assayed (■). The light-activated rate of 0.73 pmol of GTPγS bound/min is the slope of the line. This rate is approximately 12-fold greater than the rate observed in the dark. The data are from one of three similar experiments.
EcoRI/ScaI fragment from pGEM-METAB85 (nt –84 to 1024) and a ScaI/HindIII fragment from pGEM-METAB75 (nt 1025–3105) were ligated with EcoRI/HindIII digested pCMV-T7–2 (SA/SD) vector to form pCMV-2 (SA/SD)-hmGluR6. The cDNA sequence of hmGluR6 was determined completely on both strands. The construction of the pCMV-T7–2 vector was described previously (27). The pCMV-T7–2 (SA/SD) vector was constructed by digesting pCMV-T7–2 with XhoI and then blunt ending the DNA. The pCMV construct was then digested with StuI. This series of digestions removes the sequence that encodes the splice donor and splice acceptor site recognition sequences (nt 1018–1147). The remaining portion of the vector was then religated to yield pCMV-T7–2 (SA/SD).

Modification of the hmGluR6 Clone—The cDNA for the human mGluR6 was modified by the addition to the C terminus of 15 amino acids (STTVSKTETSQVAPA) corresponding to the epitope for the rho-1018–1147. The remaining portion of the vector was then religated to the splice donor and splice acceptor site recognition sequences (nt 1025–3105) and then blunt ending the DNA. The pCMV construct was then digested with StuI. This series of digestions removes the sequence that encodes the splice donor and splice acceptor site recognition sequences (nt 1018–1147). The remaining portion of the vector was then religated to yield pCMV-T7–2 (SA/SD).

Expression and Purification of hmGluR6—The hmGluR6 clone was expressed in COS-7 cells following transfection with LipofectAMINE. Cells were harvested 48 h posttransfection for purification. The transfected cells were then solubilized in 50 mM Tris buffer, pH 7.0, containing 140 mM NaCl, 1 mM dithiothreitol, 1% CHAPS, and 10 mg/ml asolectin. The solubilized proteins were purified by immunoaffinity chromatography using the bovine rhodopsin antibody, 1D4, as described by Oprian et al. (30). The purified receptor in a CHAPS asolectin solution was applied to a Sephadex G-50 column to remove the detergent and form lipid vesicles containing the purified receptor (31). The fractions containing the vesicles were pooled and concentrated by centrifugation in a Centricon-30. These vesicles were then assayed for activity, and the amount of expressed protein estimated by Western blotting using the antibody 1D4 to visualize the receptor.

Expression and Purification of Bovine Rhodopsin—Wild type bovine opsin was expressed, purified, and reconstituted into asolectin vesicles as described above for hmGluR6. Vesicles containing wild type bovine opsin were then incubated with the chromophore 11-cis-retinal (0.2 mM) in the dark for at least 1 h.

Purification of Transducin—Transducin was purified from bovine retina according to the procedure of Wessling-Resnick and Johnson (32) and then subjected to ion exchange chromatography on DE-52 as described by Baehr et al. (33).

Assay for Activation of Transducin and G o—Purified hmGluR6 in lipid vesicles was assayed for its ability to catalytically activate transducin or G o, by following the binding of [35S]GTP S as has been previously described for rhodopsin (34). Purified hmGluR6 in lipid vesicles (final concentration, 2.6 × 10⁻⁹ M) was incubated with the indicated amounts of L-AP4 in reaction buffer containing 1 mM Tris, 10 mM NaCl, 0.5 mM MgCl₂, and 10 μM EDTA, pH 7.0, at 30 °C for 30 min. The reaction was initiated by the addition of transducin or G o and GTP S resulting a final concentration of 1 μM. Aliquots were assayed at indicated times by filtering through nitrocellulose filters. Rhodopsin activation of transducin was performed as described previously (34). SDS/PAGE—SDS/PAGE was performed according to Laemmli using both 10 and 15% polyacrylamide gels (35). Proteins were visualized using silver staining according to the procedure of Bloom et al. (36).

Immunological Analysis—Immunoblot analysis was performed according to the methods of Burnette (37). Rhodopsin and the modified hmGluR6 were detected using the monoclonal antibody 1D4. Antibody binding was visualized using a radiolabeled secondary antibody that was visualized using a PhosphorImager (Molecular Dynamics).

RESULTS

A full-length human cDNA clone for mGluR6 was constructed from two overlapping cDNA fragments isolated from human retinal cDNA libraries. The percentage of identity of the human and rat mature mGluR6 protein is 94.8%, and the divergence is largely due to the divergence of the signal peptide. The four putative N-glycosylation sites and two putative PKC phosphorylation sites are conserved between the human and rat sequences. The deduced amino acid sequence of hmGluR6 reported here is 99.8% identical to the hmGluR6 sequence reported by Laurie et al. (18). The only differences occur at Arg⁵⁹ → Pro and Val⁵⁵⁷ → Ala (the sequence of Laurie et al. mutated to our sequence). The underlined residue is identical to those reported for the rat sequence (17).

Expression and Purification of hmGluR6—The cDNA clone for hmGluR6 was modified with the addition of a 15-amino acid epitope tag at the C-terminal tail, which corresponds to the epitope for the rhodopsin monoclonal antibody 1D4 (21). The strategy of adding this epitope has been utilized previously for human color visual pigments and the β₂-adrenergic receptor (26, 27). In these instances the additional amino acids did not alter the properties of the membrane proteins but allowed for easy purification using immunoaffinity chromatography (26, 27).

The hmGluR6 protein was expressed in COS-7 cells and purified by immunoaffinity chromatography using the 1D4 antibody. Using this procedure a major protein band with an apparent molecular mass of approximately 103,000 Da was visualized on an SDS/PAGE gel using silver staining (Fig. 1A, lanes

![Graph showing GTPγS binding to Gα](image)

**Fig. 3.** L-AP4 activated hmGluR6 activates rat Gα. The time course for the binding of GTPγS to Gα, as catalyzed by L-AP4-activated hmGluR6 purified from transfected COS cells and reconstituted into lipid vesicles is shown. The assay was performed as described under “Experimental Procedures.” 6 mM L-AP4 (■) was used in this assay and compared with identical reactions in the absence of agonist (○) and with 10 μM MPPG (▲), the Class III-specific antagonist. The rate of 2.1 pmol of GTPγS bound/min is the slope of the line and represents the rate of activation in the presence of agonist. This rate is approximately 5-fold greater than the rate observed in the absence of agonist and 2.6-fold greater than the rate in the presence of the inhibitor MPPG. The data are from one of three similar experiments.

| Receptor       | Transducin | Gα     | Ratio of Gα/Transducin activity |
|----------------|------------|--------|----------------------------------|
| hmGluR6 (L-AP4 = 6 mM) | 0.0574 ± 9.34 × 10⁻⁵ (n = 6) | 1.03 ± 0.62 (n = 3) | 18-fold |
| Rhodopsin      | 0.81 ± 0.123 (n = 3) | no light effect (n = 3) | 0       |

**TABLE I**

The G-protein activation by hmGluR6 and rhodopsin.

The initial rate of G-protein activation is indicated as pmol of GTPγS bound/min and was determined as described in the Fig. 2 legend. There is an intrinsic activity, agonist-insensitive activity that has not been subtracted from these rates. The rates represent the means ± S.E. of the indicated number of similar experiments.
A Go-type G-protein. To further investigate the activation of ON-bipolar cells do not contain transducin but do include

activation was 8.4 mM. May be the preferred substrate for hmGluR6.

did not show). To further investigate the specificity of the coupling of hmGluR6 to Go in this reconstitution system, we examined the ability of the Class III-specific mGlur antagonist MPPG (44) to inhibit the l-AP4-stimulated GTPγS binding. Preincubation with 10 μM MPPG in the binding assay inhibited the l-AP4-stimulated GTPγS binding by 42% (n = 3, Fig. 3 and Table II).

In summary, the hmGluR6 protein has been expressed in vitro, purified, and functionally reconstituted into an in vitro G-protein activation assay. We observed activation of two G-proteins, transducin and Go, by agonist activated hmGluR6; the activation of Go was 18-fold greater than activation of transducin.

**DISCUSSION**

In rodents, mGluR6 is nearly exclusively expressed in ON-bipolar (15). Activation of this G-protein-coupled receptor by glutamate leads to the activation of a biochemical cascade that results in the hyperpolarization of ON-bipolar cells (8, 9). Electrophysiological and pharmacological studies have implicated a cGMP biochemical cascade in this signaling pathway (10, 11). The identity of the specific G-protein activated in this system as well as the effector enzyme have yet to be conclusively identified. In an attempt to address this problem, we have cloned, expressed, purified, and reconstituted the hmGluR6 protein in an in vitro system where the coupling of the receptor to different G-proteins can be explored.

We found that hmGluR6 protein expressed in vitro and reconstituted into lipid vesicles can functionally couple to two known G-proteins. When activated by the agonist l-AP4, hmGluR6 can couple to both transducin and Go. Activation was measured by the binding of GTPγS to the G-protein. The rate of activation of Go is 18-fold greater than the rate of activation of transducin. Glutamate can also activate hmGluR6 in this reconstituted system and agonist (l-AP4) activation is inhibited by the Class III-specific antagonist MPPG. These results indicate that we are measuring functional coupling of hmGluR6 to both Go and transducin. This is the first report of the purification and functional coupling of hmGluR6 to a G-protein, or any glutamate metabotropic receptor, in an in vitro reconstitution system.

The results reported here do not identify the G-protein that actually couples to the hmGluR6 in the ON-bipolar cells. They suggest that the specificity of hmGluR6 for Go is greater than it is for transducin. This is consistent with the finding that antibodies to Go, but not transducin localize to ON-bipolar cells (19). The data presented here suggest that hmGluR6 can also activate transducin. Both Go and transducin are members of the same family of G-proteins (45). It is possible that the G-protein in ON-bipolar cells has characteristics of both Go and transducin. Alternatively, there may be multiple G-proteins activated by mGluR6 in vitro. The second messenger system
mediating the glutamate-dependent hyperpolarization in ON-bipolar cells remains to elucidated. Effectors enzymes activated by Gα are now possible candidates for involvement in this second messenger system.

Transducin is not known to be a promiscuous G-protein. It is not activated by many G-protein-coupled receptors and seems to retain some degree of specificity for visual opsins. It can be activated by the αβ-adrenergic receptor (46). The results reported here are important because they suggest that the hmGluR6 can activate transducin, the rod-specific G-protein. Because hmGluR6 is a G-protein-coupled receptor that does not have substantial sequence homology to opsin, these results suggest a novel coupling of a non-opsin G-protein-coupled receptor to transducin.

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