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Cloning and Expression of a Human Metabotropic Glutamate Receptor 1α: Enhanced Coupling on Co-transfection with a Glutamate Transporter

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SUMMARY

We cloned and expressed a human metabotropic glutamate receptor 1α (HmGluR1α) in a novel cell line. The human mGluR1α cDNA was found to be 86% identical to rat mGluR1α, and the predicted protein sequence was found to be 93% identical to rat mGluR1α. We expressed HmGluR1α in AV12–664, an adenovirus-transformed Syrian hamster cell line. To prevent tonic activation of HmGluR1α by glutamate that may be released by these cells into the extracellular medium, HmGluR1α was co-expressed in AV12–664 cells with a rat glutamate/aspartate transporter (GLAST). This allowed investigation of the effect that clearance of glutamate from the extracellular space would have on HmGluR1α function. A comparison of mRNA levels revealed that HmGluR1α was similarly expressed in cells with or without co-expression of GLAST. However, HmGluR1α-mediated phosphoinositide hydrolysis was efficiently elicited only in cells co-expressing rat GLAST. Blockade of glutamate transport by L-*trans*-pyrrolidine-2,4-dicarboxylic acid resulted in an increase in glutamate levels in the media and an increase in basal HmGluR1α-mediated phosphoinositide hydrolysis. Long-term pretreatment of cells with L-*trans*-pyrrolidine-2,4-dicarboxylic acid resulted in media glutamate levels similar to those in cells not expressing GLAST. However, this resulted in a dramatic decrease in 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid- and glutamate-stimulated phosphoinositide hydrolysis. These studies suggest that co-expression of mGluR1α with a glutamate transporter prevents desensitization of the receptor, thus achieving optimal coupling of the receptor with its effector system.

In the vertebrate central nervous system, glutamate activates two broad families of receptors: ionotropic glutamate receptors, which are ligand-gated ion channels, and mGluRs, which are linked to a number of effector systems through various G proteins. At least eight rat mGluRs have been cloned, and these receptors are further subdivided into groups according to similar sequence homology, signal transduction pathways, and pharmacological profile. For example, group 1 mGluRs consist of mGluR subtypes 1 and 5. These receptors share a 60% homology (1), have similar agonist profiles (1–4), and stimulate phosphoinositide hydrolysis in expression systems (2, 4). Both mGluR1 and mGluR5 have at least one alternative splice variant (5–7). In addition, mGluRs include group 2 (mGluR2 and mGluR3) and group 3 (mGluR4, mGluR6, mGluR7, and mGluR8) receptors. When expressed in cell lines, both group 2 and 3 mGluRs have been shown to couple to inhibition of adenylate cyclase (8–10).

We cloned a cDNA encoding HmGluR1α and found it to be 86% identical to rat mGluR1α. To determine the signal transduction properties and pharmacological profile of HmGluR1α, we stably transfected the cloned receptor into a mammalian nonneuronal cell line (AV12–664; ATCC CRL 9595) (AV12/HmGluR1α cells). Mammalian cells grown in culture require glutamate for growth and function, and most immortal cell lines are capable of producing glutamate endogenously. Substantial amounts of this endogenous glutamate may be secreted into the culture medium. The presence of significant levels of glutamate makes it difficult to express and study glutamate receptors in such cell lines. Because glutamate is the endogenous agonist of mGluRs, the release of glutamate into the extracellular medium is likely to cause persistent activation of the transfected receptor. One mechanism by which excess glutamate can be removed from the extracellular space is by an effective glutamate transport system. Although many cell lines of nonneuronal origin may possess glutamate transporter capabilities (11–13), it is not

ABBREVIATIONS: mGluR, metabotropic glutamate receptor; GLAST, glutamate/aspartate transporter; HmGluR1α, human metabotropic glutamate receptor 1α; bp, base pair(s); PCR, polymerase chain reaction; RT, reverse transcription; kb, kilobase; [^{3}H]InsP, [^{3}H]inositol monophosphate; 1*S*,3*R*-ACPD, 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid; L-CCG-I, (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine; MCPG, (+)-α-methyl-4-carboxyphenylglycine; 4C3HPG, (*RS*)-4-carboxy-3-hydroxyphenylglycine; *trans*-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; 3,5-DHPG, (*RS*)-3,5-dihydroxyphenylglycine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; SSC, standard saline citrate; SDS, sodium dodecyl sulfate.

clear which of the cloned glutamate transporters (14) may be related to the transporters expressed in these cell lines.

In the present study, we stably transfected AV12–664 cells with rat GLAST (15) to ensure the uptake of glutamate that may be released into the extracellular medium. The rat GLAST transporter has been shown to transport glutamate and aspartate with relatively high affinity (15) in a Na^+-dependent manner (15, 16). These RGT cells were subsequently transfected with HmGluR1α (RGT/HmGluR1α cell line). A series of studies was performed to test the hypothesis that co-expression of the rat GLAST transporter to facilitate uptake of glutamate from the extracellular medium enhances HmGluR1α receptor coupling by preventing receptor desensitization. Using this cell line, we also determined that the pharmacological profile of the human mGluR1α is similar to its rat counterpart.

Materials and Methods

Isolation of cDNA clones. cDNA sequences encoding HmGluR1α were isolated from a λ gt11 cDNA library (R. Neve, University of California, Santa Barbara) and a λ ZAPII library (Stratagene) derived from human fetal brain. Oligonucleotides corresponding to bp 532–585, 1339–1399, and 3420–3561 of the published rat mGluR1 cDNA sequence (3) were synthesized and used as PCR primers and/or hybridization probes under low-stringency conditions to screen the library (17). PCR amplification was performed for 35 cycles (94° for 1 min, 50° for 1 min, and 65° for 4 min). Nylon filter hybridization was performed at 50° in 1 M NaCl, 50 mM Tris, pH 7.5, 10× Denhardt's solution, 0.5% SDS, and 100 μg/ml yeast tRNA. The filters were washed with 2× SSPE (1 × = 0.15M NaCl, 0.02 M Na_2HPO_4, 0.02 M EDTA, pH 7.4) at 50°. Positive clones were isolated and characterized for size and relatedness to mGluR1, as determined by Southern blot analysis.

cDNA encoding GLAST (15) was isolated from a λ ZAPII cDNA library derived from rat hippocampus (Stratagene). The published sequence was used to design PCR primers and produce a [^{32}P] hybridization probe that was subsequently used to screen the library and isolate clones containing the entire coding sequence.

Cell cultures and transfection. The AV12–664 (AV12) cell line, previously deposited with American Type Culture Collection (accession number CRL 9595), is an adenovirus-transformed Syrian hamster cell line. To create the RGT cell line, GLAST cDNA was transfected into AV12 cells. After excision of an isolated GLAST clone into pBluescript SK^+, the coding sequence was recovered on a 2640-bp *Eco*RV-*Sma*I restriction fragment. *Xba*I linkers were added, and this fragment was cloned into the *Xba*I site of a mammalian expression vector, pRS/RSV (Invitrogen).

For expression of HmGluR1α, the cDNA sequence was inserted into a mammalian expression vector, pGT-h (18), which uses the major late promoter of adenovirus type 2 to provide high-level expression in the presence of the adenovirus E1, a gene product found in AV12–664 and RGT cells. A 4.1-kb cDNA fragment was recovered after digestion of a λ clone with *Eco*R1 restriction endonuclease. *Eco*R1-*Bam*H1 adaptors (New England Biolabs) were added, and the fragment was ligated into the *Bcl*I site downstream of the adenovirus promoter in the pGT-h expression vector.

Transfections of the plasmids into cells were carried out using a modified calcium phosphate precipitation method (19) with reagents obtained from Stratagene. Ten micrograms of plasmid were used without carrier DNA for each 10-cm petri plate of cells at ~50% confluency. Clones expressing GLAST were selected by resistance to G-418 (500 μg/ml) (GIBCO-BRL). HmGluR1α-expressing clones were selected by resistance to hygromycin (250 μg/ml). Resistant clones were tested for HmGluR1α expression by measurement of agonist-stimulated phosphoinositide hydrolysis. Both AV12/HmGluR1α cells and RGT/HmGluR1α cells were grown in Dulbecco's modified Eagle's medium supplemented with 1.2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 5% dialyzed fetal calf serum, and 250 μg/ml hygromycin. In addition, media for RGT cell lines contained 500 μg/ml G418. Cells were maintained in a 6.8% CO_2 incubator at 37°.

Preparation of internal standard cRNA. HmGluR1β cRNA was used as an internal standard in quantifying mRNA levels from the various cell lines. HmGluR1β in a pBluescript II SK^+ plasmid was linearized by cutting at an *Xba*I restriction site downstream from the 3′ end of the HmGluR1β DNA. Linearized template was phenol/chloroform extracted with equal volumes of phenol and $CHCl_3$/isoamyl alcohol (24:1) and recovered by ethanol precipitation. cRNA was synthesized from approximately 1 μg of the linearized product using T7 polymerase and an *in vitro* transcription system (Stratagene). The transcription product was treated with DNase I (GIBCO-BRL) to remove plasmid DNA. The HmGluR1β cRNA was phenol/chloroform extracted and ethanol precipitated.

Isolation of RNA from cell lines. Total RNA was isolated from four cell lines: (a) AV12 cells, (b) RGT cells (AV12 cells transfected with rat GLAST transporter), (c) AV12/HmGluR1α cells, and (d) RGT/HmGluR1α cells. RNA was isolated from cells grown to confluency using a modification of the phenol-guanidinium isothiocyanate method (20). Briefly, cells grown in monolayer were lysed with TRIzol reagent (GIBCO-BRL); the mixture was centrifuged; and the aqueous phase containing RNA was recovered. RNA was precipitated from the aqueous phase with isopropyl alcohol. The RNA pellet was washed with 75% ethanol and dissolved in DEPC-treated water. Total RNA from each of the cell lines was subsequently treated with DNase I (amplification grade; GIBCO-BRL) to degrade any contaminating DNA. DNase I was removed by phenol/chloroform extraction, and RNA was recovered by precipitation with 2.5 volumes of ethanol from 0.3 M sodium acetate, pH 6. RNA was dissolved in DEPC-treated water, and the concentration was determined by measuring the optical density at 260 nm.

Relative quantification of HmGluR1α mRNA levels. RT-PCR was performed with a Stratascript RT-PCR kit (Stratagene). For RT of RNA, 2.5 μg of total RNA was used from each of the four samples of RNA isolated. Samples of RNA from AV12/HmGluR1α and RGT/HmGluR1α were spiked with decreasing amounts of HmGluR1β cRNA. RT was performed with random hexamers as primers. Products from this reaction were used for PCR analysis. Each of the RT products (3 μl) was added to a PCR mix (50 μl total volume) to give final concentrations of 10 mM Tris·HCl, pH 8.5, 30 mM KCl, 3 mM $MgCl_2$, 0.001% gelatin, 400 μM deoxynucleoside-5′-triphosphates, and 20 pmol each of a primer pair designed to amplify both HmGluR1α and HmGluR1β. The sequence of the 5′ primer (5′ to 3′; from 5′ position 2719) was GTG CAT GTT CAC TCC CAA GAT GTA CAT. The sequence of the 3′ primer (5′ to 3′; to 3′ position 3156) was CAC GCG CCT GTG CAC CAC CAT GGA AG. The mixtures were heated to 94° for 5 min and then cooled to 60°, at which time *Taq* DNA polymerase (0.4 μl in 5 μl water; GIBCO-BRL) was added to each tube. The samples were then amplified for 35 cycles (94° for 1 min, 60° for 1 min, and 72° for 2 min). This was followed by a 7-min elongation. Each product was diluted 1:50 and was added to a fresh PCR mix and amplified in a similar manner for two additional cycles to eliminate heterodimers (21). Products from the final PCR were analyzed by electrophoresis on a 1.5% (wt/vol) agarose gel. DNA was blotted onto nylon membrane (Bio-Rad) and fixed by ultraviolet light (UV Stratalinker 1800, Stratagene). An oligonucleotide probe common to both HmGluR1α and HmGluR1β was phosphorylated with [^{32}P]ATP (New England Nuclear) using a T4 phosphonucleotide kinase (Boehringer Mannheim). The sequence of this probe (5′ to 3′; to 3′ position 2896) was AAG ATT ACC GTT CAG ACA CAG. The membrane-bound DNA was hybridized with the [^{32}P]-labeled oligonucleotide probe at 50° for 16–24 hr in a solution containing 5× SSC, 20 mM Na_2HPO_4, pH 7.2, 7% SDS, 10× Denhardt's solution, and 100 μg/ml denatured tRNA. The blot was washed twice for 30 min in a

solution containing 3× SSC, 10× Denhardt's solution, 5% SDS, and 25 mM Na_2HPO_4, pH 7.2. A final wash was carried out at 50° for 30 min with a solution of 1× SSC and 1% SDS. The membrane was exposed to X-ray film (Amersham Hyperfilm-MP) for 2–24 hr for visualization. Radioactivity was quantified with a Molecular Dynamics PhosphorImager SI in conjunction with an ImageQuant software analysis program.

Measurement of phosphoinositide hydrolysis. Cells were plated into 24-well plates and grown for 1–4 days in normal growth media. This was followed by a medium change in which 1 ml of fresh media containing 4 μCi/ml [2-^{3}H]*myo*-inositol (Amersham) was added to each well. After 18–24 hr, medium was removed, and the cells were washed twice with Dulbecco's modified Eagle's medium containing 10 mM *myo*-inositol, 10 mM LiCl, and 10 mM HEPES. Agonists were added to the medium, and cells were incubated at 37° for 1 hr under a CO_2 atmosphere. Antagonists or uptake inhibitors, when used, were added 20 min before agonists, except in experiments in which cells were preincubated with *trans*-PDC for 24 hr. At the end of the incubation, the reaction was stopped by placing the plates on ice, quickly removing the incubation media, and adding 1 ml acetone/methanol (1:1). Stimulation of phosphoinositide hydrolysis was assayed by measuring the accumulation of [^{3}H]InsP. [^{3}H]InsP was isolated by QMA Sep-Pak anion column chromatography (Millipore Corp.) by elution with triethylammonium bicarbonate (Fluka Chemicals) (22). Data were calculated as dpm of [^{3}H]InsP/milligram of protein and converted to a percentage of the basal [^{3}H]InsP value in each experiment. Protein content in each well was determined with a modified Bradford-Pierce assay (Pierce Chemicals).

Measurement of glutamate uptake. Cells were plated into 12-well plates and grown for 1–3 days in normal growth media. The culture medium was exchanged with PBS containing 138 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.5 mM $MgCl_2{\cdot}6H_2O$, and 8.1 mM $Na_2HPO_4{\cdot}7H_2O$. The cells were preincubated for approximately 5 min at 37° under a CO_2 atmosphere. Subsequently, this medium was exchanged with 0.5 ml PBS containing 100 nM L-[3,4-^{3}H]glutamate (New England Nuclear) diluted with cold substrate to obtain a desired final concentration of 30 μM. This concentration of glutamate was near the K_m value (52 μM) of the GLAST transporter determined in preliminary experiments with RGT/HmGluR1α cells. Triplicate assays were performed with increasing concentrations of *trans*-PDC or mGluR1α agonists, and the incubation was carried out for 10 min. Reactions were stopped by quickly placing the plates on ice, removing the incubation medium, and washing with ice-cold PBS containing excess unlabeled glutamate. Cells were solubilized in 2 ml of 0.25 M NaOH at 37° for 30 min. Aliquots were used to measure radioactivity and protein content (Pierce Chemicals). To determine nonspecific uptake, assays were performed at 4° for 10 min. Nonspecific uptake was found to be <3% of uptake at 37°, and all samples were corrected for this value.

HPLC analysis of glutamate concentrations. Medium from wells plated with AV12/HmGluR1α cells or RGT/HmGluR1α cells was collected for HPLC analysis after the 60- or 80-min incubation with agonists/antagonists. In the experiments in which cells were exposed to *trans*-PDC for 18–24 hr, the medium was collected before incubation with agonists. Samples of medium were diluted with volumes of 0.01 N HCl to give concentrations in the range of glutamate standard solutions (0.1–10 μM). The samples were mixed with equal volumes of Fluo-R fluorescence reagent (Beckman Instruments). The mix was kept at room temperature for 1 min to derivatize the sample before being injected into a 20-μl loop by a Beckman Autosampler 507. The column used was an Ultrasphere ODS C18 5-μm column (2 mm × 25 mm) at 30°. The HPLC system consists of a high-pressure pump (Beckman System Gold) with a flow rate of 0.3 ml in coordination with a fluorescence detector (Perkin Elmer LC-240; excitation/emission wavelengths, 360/450 nm). The mobile phase consisted of (A) 50 mM sodium phosphate, pH 7.2, containing 2% methanol and (B) methanol. Gradient elution involved using 95% A/5% B initially and then increasing to 20% B over 20 min. Mobile phase B was increased to 70% over 3 min, and maintained for 15 min to elute other substances, and then returned to the initial conditions for 20 min before running the next sample. Based on comparison to standards, glutamate eluted at 16 min.

Statistical analysis. To determine EC_{50} values from concentration-response curves on [^{3}H]InsP accumulation, we used the median-effect plot of Chou and Talalay (23). Statistical significance was determined by using one-way analysis of variance in conjunction with a Dunnett's test or two-way analysis of variance in conjunction with least-squares means. Values of $p < 0.05$ were considered significantly different from control.

Materials. The following items were purchased from Tocris Cookson: 1S,3R-ACPD, L-CCG-I, quisqualic acid, MCPG, 4C3HPG, L-2-amino-4-phosphonobutyric acid, and *trans*-PDC. 3,5-DHPG was supplied by S. Richard Baker (Lilly Research Centre, Windlesham, UK). All materials for cell culture media were purchased from GIBCO-BRL.

Results

cDNA cloning of human mGluR1. One of the λ cDNA clones contained a 4.1-kb insert and was shown by DNA sequencing to contain a long, open reading frame highly homologous to rat mGluR1α. Another clone, identical to HmGluR1α but containing an 85-bp insert at the corresponding location like that found in rat mGluR1β (5), was also isolated.

The HmGluR1α cDNA contains an open reading frame of 3582 bp, predicting a protein of 1194 amino acids, whereas the rat mGluR1α open reading frame is 3597 bp (1199 amino acids). The two cDNA sequences were found to be 86% identical. In comparing the predicted polypeptide sequence of the human versus rat mGluR1α, we found them to be 93% identical (Fig. 1). As expected, the sequences of the transmembrane regions and cytoplasmic loops are highly conserved. The amino-terminal domain, which is proposed to contain the agonist binding site, is the next most conserved region. The greatest divergence in the protein sequences occurs within the carboxyl-terminal cytoplasmic domain and includes deletion of seven residues within the unusual proline/glutamine stretch found in mGluR1α. All of the sites in rat mGluR1α proposed for *N*-glycosylation and seven of eight proposed sites for phosphorylation are conserved in HmGluR1α. Human mGluR1β differs from HmGluR1α by an additional 85-bp insert at position 2660 of the HmGluR1α cDNA coding sequence. Sequence comparison with rat mGluR1β revealed that the sequence of this insert is 100% identical in the rat and human cDNAs (sequence not shown).

We measured HmGluR1α mRNA levels in both AV12 and RGT cell lines using a modified version of the PATTY technique (21) with HmGluR1β cRNA as an internal standard. A specific primer pair was designed that would encompass the 85-bp insertion in HmGluR1β at the carboxyl terminal, and DNA bands were probed by a [^{32}P]-labeled oligonucleotide probe common to both HmGluR1α and HmGluR1β (Fig. 2, *top*). By comparing radioactivity in the HmGluR1α DNA bands with their HmGluR1β internal standard bands (Fig. 2, *bottom*), we determined that mRNA levels in RGT/HmGluR1α cells were 1.4 times the mRNA levels in AV12/HmGluR1α cells.

Having determined that AV12/HmGluR1α and RGT/HmGluR1α cell lines had relatively similar mRNA levels, we compared HmGluR1α-mediated phosphoinositide hydrolysis in both cell lines. This allowed determination of the effects of

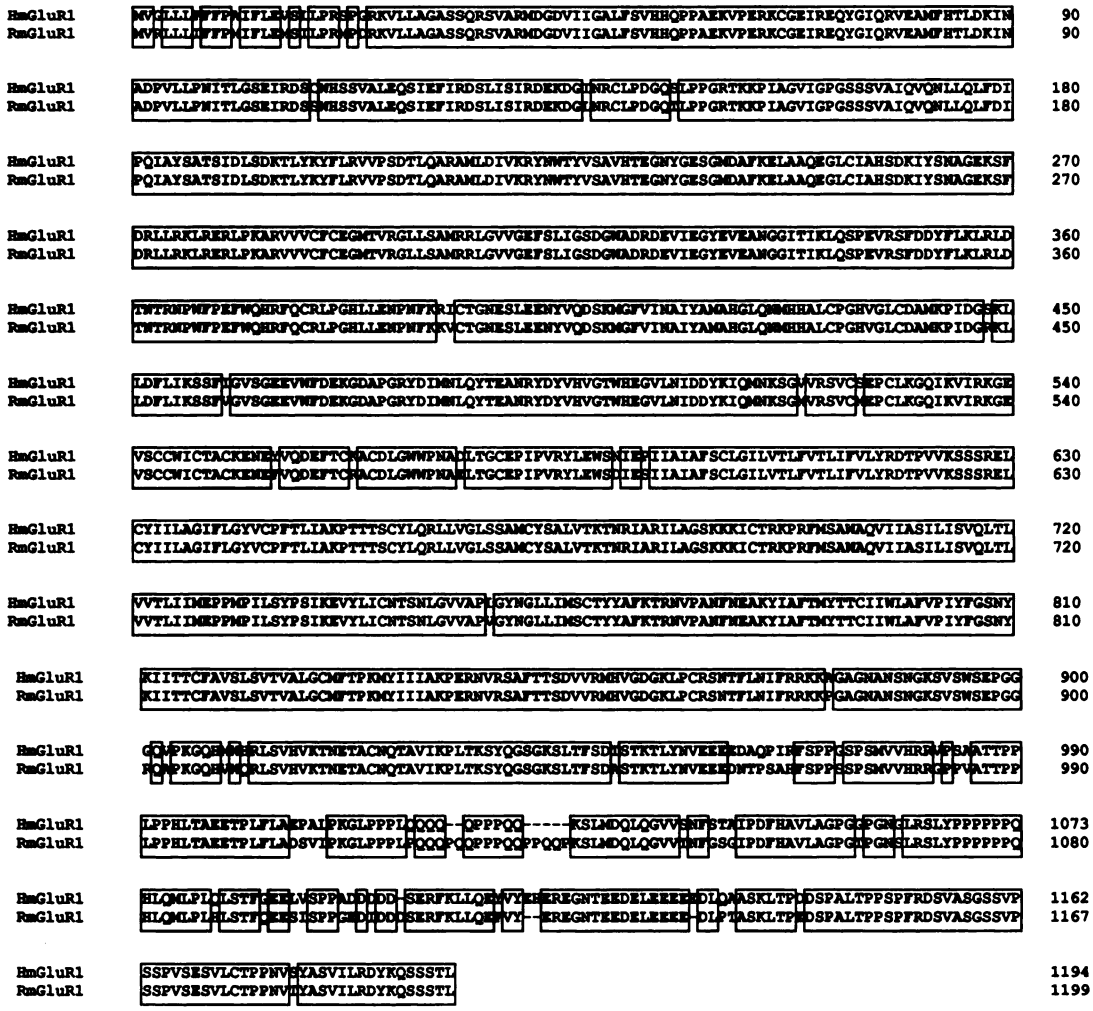

Fig. 1. Amino acid sequences of the coding regions for human (*top*) versus rat (*bottom*) mGluR1α. *Boxed regions*, regions that are identical in rat mGluR1α and HmGluR1α. *Dashes*, missing amino acids. The GenBank accession number for the nucleotides sequence of human mGluR1α is U31215. For human mGluR1β, the nucleotide sequence (not shown) was also deposited in GenBank (accession number U31216).

removal of glutamate from the extracellular medium on HmGluR1α receptor coupling. Fig. 3 shows the percent stimulation of phosphoinositide hydrolysis in AV12 cells transfected with HmGluR1α (AV12/HmGluR1α) and in cells co-transfected with HmGluR1α and the rat GLAST transporter gene (RGT/HmGluR1α). We found that glutamate markedly stimulated phosphoinositide hydrolysis in RGT/HmGluR1α cells. The EC_{50} for glutamate-stimulated phosphoinositide hydrolysis in RGT/HmGluR1α cells was 61.0 ± 26.3 μM. In contrast, glutamate only slightly stimulated phosphoinositide hydrolysis in AV12/HmGluR1α cells (Fig. 3). Because HmGluR1α was much more efficiently coupled to phosphoinositide hydrolysis in RGT/HmGluR1α cells, we used this cell line to determine the pharmacological profile of HmGluR1α.

Pharmacological profile of HmGluR1α. The phosphoinositide hydrolysis response to a number of mGluR agonists in RGT/HmGluR1α cells was examined as a comparison with the known pharmacology of the rat receptor. Consistent with the pharmacological profile of group 1 rat mGluRs, we found that the order of potency of agonists was quisqualate ≫ 3,5-DHPG > L-CCG-I ≥ 1*S*,3*R*-ACPD (Fig. 4). These agonists had EC_{50} values of 0.35 ± 0.02, 10.0 ± 1.2, 31.7 ± 3.7, and 36.1 ± 3.4 μM, respectively. Neither the inactive isomer of ACPD, 1*R*,3*S*-ACPD, nor the group 3-preferring agonist, L-2-amino-4-phosphonobutyric acid, stimulated phosphoinositide hydrolysis in HmGluR1α (Fig. 4).

To examine whether direct-acting mGluR1α agonists interact at the GLAST transporter and verify inhibition of glutamate transport by *trans*-PDC, we measured [^{3}H]glutamate uptake by RGT/HmGluR1α cells. Uptake as a function of substrate concentration was fitted to the Michaelis-Menten equation. Glutamate uptake was saturable with a K_m of ~52 μM (n = 2). This value is similar to the reported K_m for the GLAST transporter in *Xenopus* oocytes (15). Thus, we determined the effect of *trans*-PDC or mGluR1α agonists on the uptake of 30 μM glutamate. We found that increasing concentrations of *trans*-PDC inhibited glutamate uptake in a concentration-dependent manner. The IC_{50} for *trans*-PDC inhibition of glutamate uptake was determined to be 74.65 ± 6.1 μM. In contrast, none of the agonists that stimulated HmGluR1α-mediated phosphoinositide hydrolysis were found to inhibit glutamate uptake in RGT/HmGluR1α cells (Table 1) at concentrations up to those that maximally stimulate phosphoinositide hydrolysis.

Recently, several phenylglycine compounds have been reported to inhibit rat mGluR1α-mediated phosphoinositide hydrolysis (24, 25). We therefore determined whether these compounds would similarly inhibit human mGluR1α-medi-

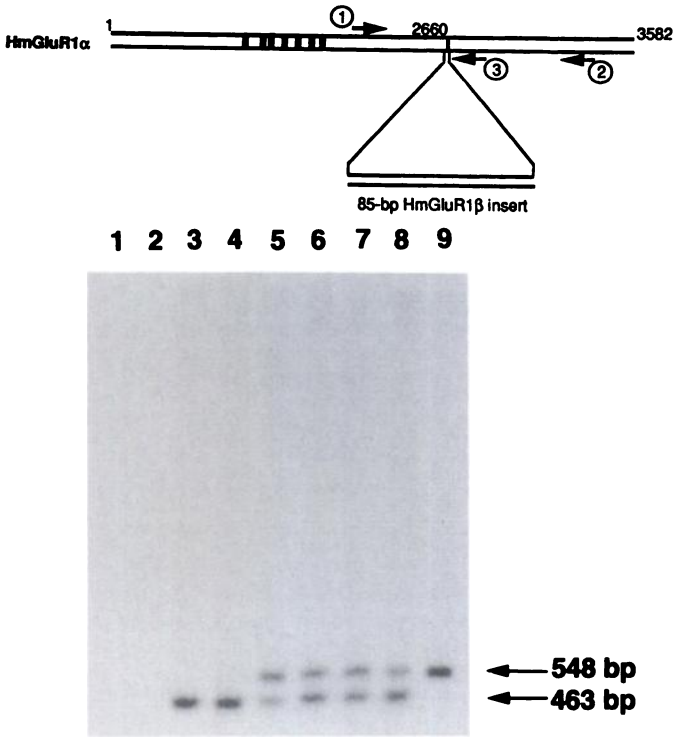

Fig. 2. HmGluR1α mRNA levels in AV12 and RGT cell lines as determined by RT-PCR. *Top*, schematic of the HmGluR1 cDNA and of the location of the 85-bp insert in HmGluR1β. *Arrows 1 and 2*, primer pair used for PCR. *Arrow 3*, probe used to label DNA bands. DNA blot analysis shows bands labeled by the probe, which was common to both HmGluR1α and HmGluR1β. *Top bands in each lane*, levels of HmGluR1β internal standard. *Bottom bands*, levels of HmGluR1α. *Lane 1*, total RNA from AV12 cells; *lane 2*, total RNA from RGT cells; *lane 3*, total RNA from AV12/HmGluR1α cells; *lane 4*, total RNA from RGT/HmGluR1α cells; *lane 5*, total RNA from AV12/HmGluR1α cells spiked with 54.6 pg HmGluR1β cRNA; *lane 6*, total RNA from RGT/HmGluR1α cells spiked with 54.6 pg HmGluR1β cRNA; *lane 7*, total RNA from AV12/HmGluR1α cells spiked with 27.3 pg HmGluR1β cRNA; *lane 8*, total RNA from RGT/HmGluR1α cells spiked with 27.3 pg HmGluR1β cRNA; and *lane 9*, 27.3 pg HmGluR1β cRNA alone.

ated phosphoinositide hydrolysis. We found that MCPG inhibited glutamate-stimulated phosphoinositide hydrolysis in a concentration-dependent manner in RGT/HmGluR1α cells (Fig. 5A). Similarly, we found that 4C3HPG inhibited 30 μM glutamate-stimulated phosphoinositide hydrolysis in a concentration-dependent manner in RGT/HmGluR1α cells; however, it was not very effective at inhibiting phosphoinositide hydrolysis by 100 μM glutamate, except at the highest concentration of 4C3HPG examined (Fig. 5B). Both of the putative antagonists examined inhibited phosphoinositide hydrolysis elicited by 30 μM glutamate more effectively than that elicited by 100 μM glutamate. These data are consistent with their actions as competitive antagonists of rat mGluR1α.

Effect of inhibition of glutamate uptake on phosphoinositide hydrolysis and extracellular glutamate levels in RGT/HmGluR1α cells. Glutamate uptake can be inhibited by *trans*-PDC, a reported potent and selective competitive inhibitor of high affinity glutamate transport (26). In our studies, the effect of increasing concentrations of *trans*-PDC on 100 μM glutamate-stimulated phosphoinositide hydrolysis in RGT/HmGluR1α cells was determined (Fig. 6A). *Trans*-PDC was added to cells 20 min before the addition of 100 μM glutamate. Increasing concentrations of *trans*-PDC

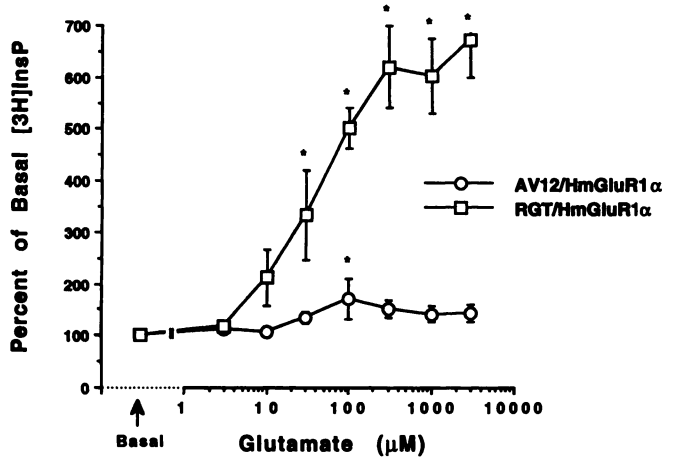

Fig. 3. Comparison of glutamate-stimulated phosphoinositide hydrolysis in AV12/HmGluR1α and RGT/HmGluR1α cell lines. Effect of increasing concentrations of glutamate on phosphoinositide hydrolysis in AV12/HmGluR1α and RGT/HmGluR1α was evaluated by determining the formation of [^{3}H]InsP (in the presence of LiCl) in cells labeled with [^{3}H]inositol. Data are expressed as percentage of basal phosphoinositide hydrolysis in each experiment. Each point represents a mean of four separate experiments, except the 3 μM point, which represents a mean of three experiments; each was done in triplicate. Mean absolute values for basal phosphoinositide hydrolysis in these experiments were 71,329 $\pm$ 12,807 dpm/mg protein in AV12/HmGluR1α cells and 132,022 $\pm$ 42,397 dpm/mg protein in RGT/HmGluR1α cells. Vertical bars represent standard error. *, $p < 0.05$ compared with basal.

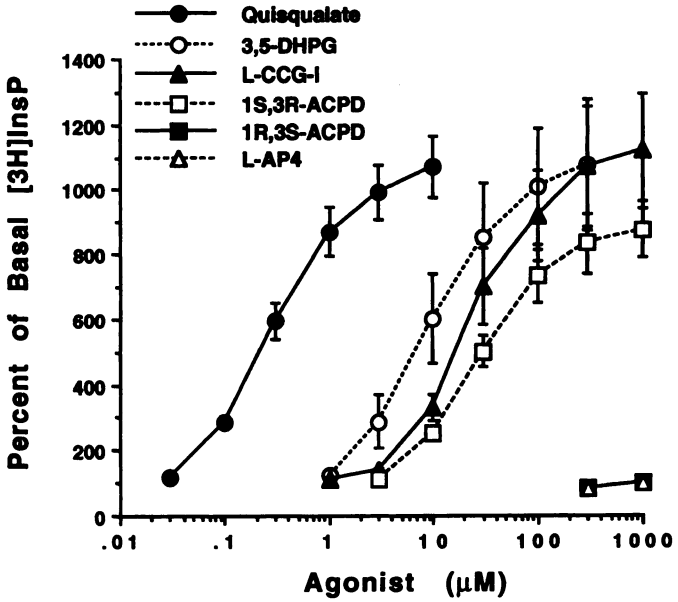

Fig. 4. Agonist-stimulated phosphoinositide hydrolysis in RGT/HmGluR1α. Phosphoinositide hydrolysis was measured as in Fig. 3. Data are expressed as percentage of basal phosphoinositide hydrolysis in each experiment. Each point represents a mean of four to six separate experiments, each done in triplicate. Vertical bars represent standard error.

alone elicited a concentration-dependent increase in phosphoinositide hydrolysis (Fig. 6A). However, in the presence of 100 μM glutamate, *trans*-PDC did not significantly increase HmGluR1α-mediated phosphoinositide hydrolysis above levels stimulated by glutamate alone (Fig. 6A).

To further characterize the effect of *trans*-PDC inhibition of glutamate uptake in RGT/HmGluR1α cells, we determined

TABLE 1

Effect of *trans*-PDC and mGluR1α agonists on uptake of [^{3}H]glutamate by RGT/HmGluR1α cells

Glutamate uptake was measured in RGT/HmGluR1α cells with 30 μM [^{3}H]glutamate. *trans*-PDC or mGluR1α agonists were added to the medium, and incubation proceeded for 10 min. Nonactive uptake (measured as glutamate uptake at 4°C for 10 min) was subtracted from each value. Nonactive uptake was less than 3% of control uptake values for each experiment (n = 3).

Treatment	Glutamate uptake	
	nmol/mg/10 min	*% of control*
Control	15.86 ± 0.79	100
trans-PDC		
30 μM	10.92 ± 0.40[a]	68.92 ± 1.11[a]
100 μM	6.72 ± 0.02[a]	42.58 ± 2.37[a]
300 μM	3.71 ± 0.02[a]	23.53 ± 1.14[a]
3,5-DHPG		
10 μM	16.12 ± 0.94	101.53 ± 1.38
100 μM	15.46 ± 0.73	97.46 ± 0.50
1S,3R-ACPD		
10 μM	15.40 ± 0.48	97.31 ± 2.95
100 μM	14.94 ± 0.54	94.30 ± 1.35
Quisqualate		
1 μM	15.52 ± 0.34	98.16 ± 3.18
10 μM	14.84 ± 0.49	93.78 ± 2.65
L-CCG-I		
10 μM	14.49 ± 0.37	91.67 ± 3.09
100 μM	14.60 ± 0.32	92.32 ± 2.92

[a] $p < 0.05$ compared with control.
Values are mean ± standard error.

the concentration of glutamate in the extracellular medium of cells used in the phosphoinositide hydrolysis experiment described above. Medium was collected at the end of the incubation with glutamate and with or without *trans*-PDC, and the concentration of glutamate was determined by HPLC analysis. On addition of 100 μM glutamate and incubation for 60 min, the media concentration of glutamate dropped to 61.9 ± 3.0 μM in the absence of *trans*-PDC (Fig. 6B). The addition of increasing concentrations of *trans*-PDC alone to the medium caused an incremental rise in the concentration of glutamate in the extracellular medium (Fig. 6B), which would account for the increased stimulation of phosphoinositide hydrolysis (Fig. 6A). The glutamate concentration in the extracellular medium of RGT/HmGluR1α cells incubated with 100 μM glutamate reached 101.6 ± 1.7 μM in the presence of 300 μM *trans*-PDC (Fig. 6B). Thus, it is likely that *trans*-PDC at a concentration of 300 μM fully inhibits glutamate transport in RGT/HmGluR1α cells.

We next determined the effect of 300 μM *trans*-PDC on phosphoinositide hydrolysis elicited by increasing concentrations of glutamate. The addition of 300 μM *trans*-PDC to the medium resulted in marked stimulation of phosphoinositide hydrolysis, even in the absence of glutamate (Fig. 7A). Furthermore, the effects of submaximal stimulatory concentrations of glutamate (10 and 30 μM) were masked by the enhancement of phosphoinositide hydrolysis induced by 300 μM *trans*-PDC. The response to a near-maximal stimulatory concentration of glutamate (100 μM) was not significantly different in the presence or absence of 300 μM *trans*-PDC (Fig. 7A). Measurement of glutamate concentrations in the extracellular medium revealed that 300 μM *trans*-PDC maintained glutamate levels approximately equal to those added exogenously to the medium, whereas glutamate concentrations were lower than the amount added exogenously in the absence of *trans*-PDC (Fig. 7B). These data support the conclusion that glutamate is effectively removed from the extracellular medium in RGT/HmGluR1α cells and that this uptake is fully inhibited by 300 μM *trans*-PDC.

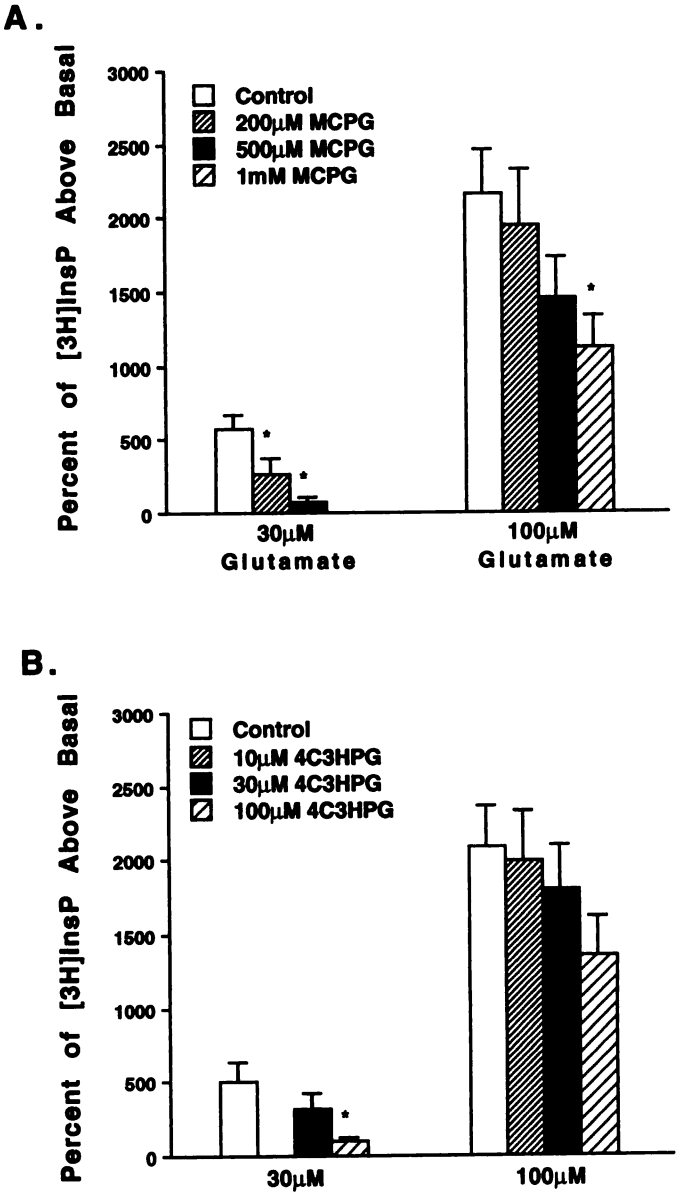

Fig. 5. A, Effect of antagonist MCPG on glutamate-stimulated phosphoinositide hydrolysis in RGT/HmGluR1α cells. B, Effect of antagonist 4C3HPG on glutamate-stimulated phosphoinositide hydrolysis in RGT/HmGluR1α cells. Phosphoinositide hydrolysis was measured as in Fig. 3. Antagonists, when used, were added 20 min before the addition of glutamate. Data are expressed as percentage above basal phosphoinositide hydrolysis in each experiment. Each bar represents a mean of three experiments, each done in triplicate. The data are presented as mean ± standard error. *, $p < 0.05$ compared with control, using one-way analysis of variance in conjunction with Dunnett's test.

Previous studies have shown that mGluRs coupled to phosphoinositide hydrolysis undergo homologous desensitization in cultures of rat cerebellar neurons (27). Thus, we tested the hypothesis that the lack of glutamate-stimulated phosphoinositide hydrolysis in AV12/HmGluR1α cells may be due to desensitization of the receptor on preexposure to glutamate in the extracellular medium. We compared the effect of preexposure to *trans*-PDC (to increase media glutamate before the phosphoinositide assay) on agonist-stimulated phosphoinositide hydrolysis in both AV12/HmGluR1α and RGT/

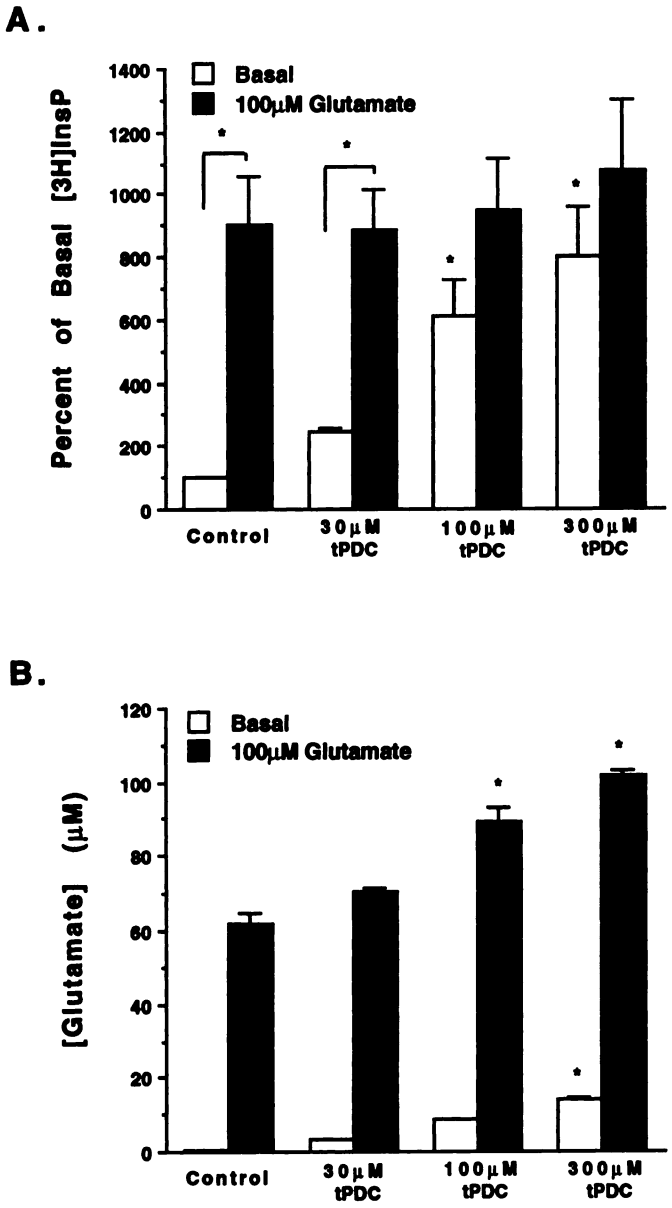

Fig. 6. Effect of increasing concentrations of *trans*-PDC on phosphoinositide hydrolysis and on extracellular glutamate levels in RGT/HmGluR1α cells. A, Increasing concentrations of *trans*-PDC were added to the media 20 min before the addition of 100 μM glutamate. On the addition of glutamate, incubation of the cells continued for an additional 60 min. Phosphoinositide hydrolysis was measured as in Fig. 3. Data are expressed as percentage of basal phosphoinositide hydrolysis in each experiment and presented as mean ± standard error. Each bar represents the mean of three experiments, each done in triplicate. *, $p < 0.05$ compared with corresponding treatment or when comparing treatments in brackets. B, Media from each well in A were collected at the end of the incubation, and glutamate concentrations were determined by HPLC analysis. Glutamate levels were determined for two of the three experiments in A, with each condition in triplicate. *, $p < 0.05$ compared with corresponding treatment.

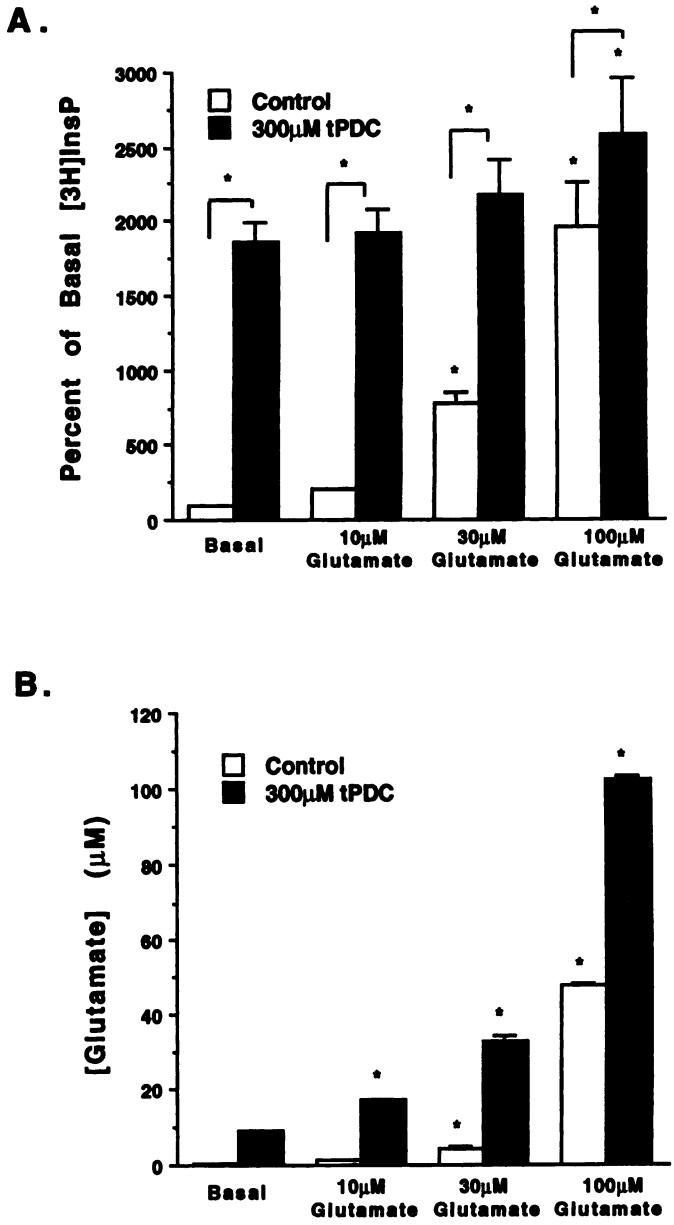

Fig. 7. Effect of *trans*-PDC on the glutamate concentration-response curve. A, Glutamate elicited a concentration-dependent increase in phosphoinositide hydrolysis. Prior incubation with *trans*-PDC for 20 min maximally stimulated phosphoinositide hydrolysis to levels achieved by the addition of 100 μM glutamate in the absence of *trans*-PDC. Phosphoinositide hydrolysis was measured as in Fig. 3. Data are expressed as percentage of basal phosphoinositide hydrolysis in each experiment and presented as mean ± standard error. Each bar represents the mean of three experiments, each done in triplicate. *, $p < 0.05$ compared with corresponding treatment or when comparing treatments in brackets. B, Media from each well in A were collected at the end of the incubation, and glutamate concentrations were determined by HPLC analysis. The addition of 300 μM *trans*-PDC restores glutamate concentrations in the media to those added exogenously. Glutamate levels were determined for each of the three experiments in A, with each condition in triplicate. *, $p < 0.05$ compared with corresponding treatment.

HmGluR1α cells. *trans*-PDC (300 μM) was added to the extracellular medium 18–24 hr before measurement of phosphoinositide hydrolysis and was washed out before the addition of agonists. We found that pretreatment of AV12/HmGluR1α cells with 300 μM *trans*-PDC did not affect the lack of stimulation of phosphoinositide hydrolysis by 1*S*,3*R*-ACPD or glutamate seen in this cell line (Fig. 8A). These results would be expected because AV12/HmGluR1α cells lack the GLAST transporter and *trans*-PDC does not appear to directly interact at mGluRs (28). In contrast, pretreatment of RGT/HmGluR1α cells with 300 μM *trans*-PDC caused a marked decrease in phosphoinositide hydrolysis by both 1*S*,3*R*-ACPD

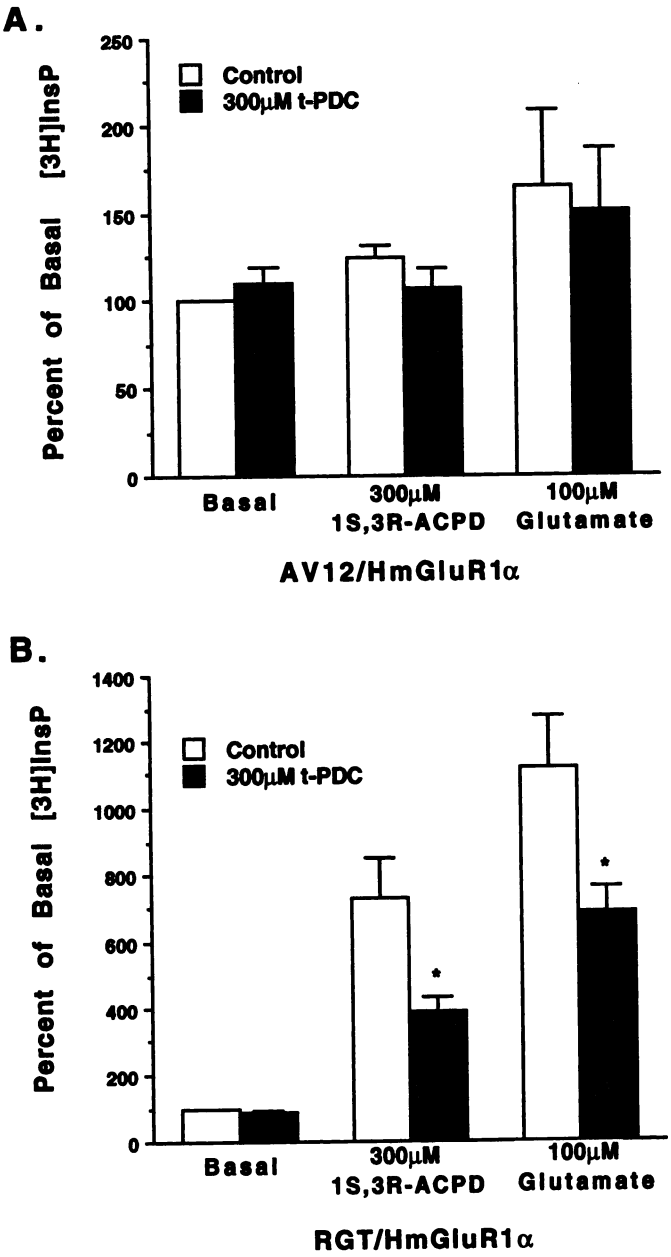

Fig. 8. Comparison of agonist-stimulated phosphoinositide hydrolysis in AV12/HmGluR1α (A) and RGT/HmGluR1α (B) cells after pretreatment with *trans*-PDC. *trans*-PDC (300 μM) was added to cells 18–24 hr before the experiment. Before the addition of agonists, cells were washed twice to remove *trans*-PDC. Phosphoinositide hydrolysis was measured as in Fig. 3. Data are expressed as percentage of basal phosphoinositide hydrolysis in each experiment and presented as mean ± standard error. Each bar represents the mean of three experiments, each done in triplicate. *, $p < 0.05$ compared with control.

and glutamate (Fig. 8B). Consistent with this, we found that pretreatment of RGT/HmGluR1α cells with 300 μM *trans*-PDC for 18–24 hr raised glutamate concentrations in the extracellular medium (Fig. 9). The significant decrease in phosphoinositide hydrolysis in RGT/HmGluR1α, combined with the rise in extracellular glutamate, suggests that lack of receptor coupling in AV12/HmGluR1α cells may be the result of desensitization in response to accumulation of high glutamate levels in the medium.

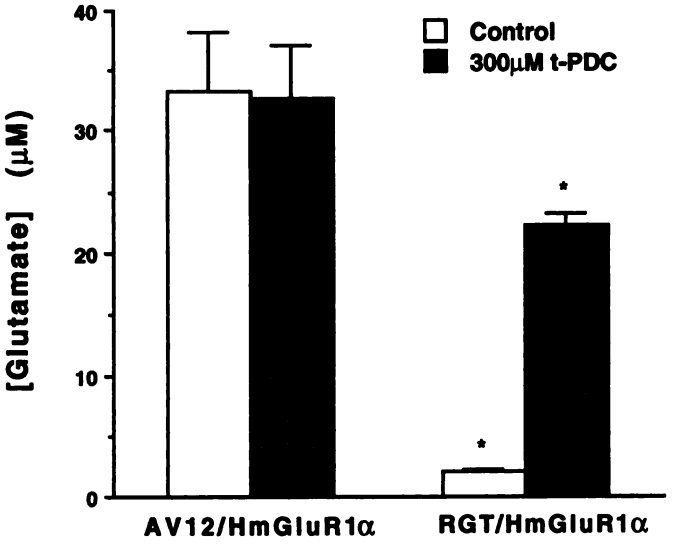

Fig. 9. Effect of pretreatment with *trans*-PDC on glutamate concentrations in AV12/HmGluR1α and RGT/HmGluR1α cells. Media were collected after the 18–24 hr pretreatment in the presence or absence of 300 μM *trans*-PDC, and glutamate concentrations were determined by HPLC analysis. Glutamate levels were determined for each of the three experiments in Fig. 8, with each condition in triplicate. *, $p < 0.05$ compared with corresponding treatment.

Discussion

We report the cloning and characterization of human mGluR1α cDNA. The sequence of the human receptor was highly homologous to the rat mGluR1α cDNA reported previously (2, 3). Both rat mGluR1 and mGluR5 cDNAs have been found to exist as splice variants (5–7). In the case of mGluR1, the inclusion of additional exons containing in-frame stop codons leads to mGluR1β or mGluR1c. These truncated receptor proteins are identical to mGluR1α from the amino terminus to Asn-887, at which point the sequences diverge. Where mGluR1α has 313 amino acids in the carboxyl terminal, this sequence is replaced by 20 or 10 different amino acids in mGluR1β and mGluR1c, respectively. We have isolated an alternatively spliced human variant, HmGluR1β, that is analogous in structure to rat mGluR1β. We found 100% identity between the rat and human mGluR1β cDNA 85-bp insert and, as in the case of the rat protein, the presence of an in-frame stop codon would be expected to result in a truncated protein of 906 amino acids. In contrast, inclusion of an additional exon (96 bp) in mGluR5 leads to a longer form, mGluR5β, which is identical to mGLuR5α except for an insertion of 32 additional amino acids. It has also been reported for this receptor that the sequence of the 96-bp insert in the cDNA is identical for rat and humans (7).

HmGluR1α in mammalian cells was expressed to study its pharmacology. However, a number of mammalian cells have been shown to release glutamate into their extracellular medium as a result of metabolic processes. This can be problematic when these cells are used as an expression system for the study of receptors whose endogenous agonist is glutamate. Thus, in the present study, we created a cell system (RGT) in which extracellular glutamate is removed from the media by a glutamate transporter, GLAST (15). The pharmacology of HmGluR1α in an AV12 cell line and in AV12 cells co-transfected with the rat GLAST transporter (RGT cells) was then studied. It was shown that the removal of glutamate from the

extracellular medium by the rat GLAST transporter in RGT/HmGluR1α cells, as opposed to the AV12 cell line lacking the GLAST transporter, resulted in efficient coupling of HmGluR1α to the phosphoinositide hydrolysis pathway.

Using the RGT/HmGluR1α cell line to perform pharmacological experiments, we found that the human mGluR1α has a signal transduction pathway and a pharmacological profile that is similar to its rat counterpart. The agonist profile was similar to that reported for rat mGluR1α, with quisqualate being the most potent agonist examined. The EC_{50} values for quisqualate and 1*S*,3*R*-ACPD in RGT/HmGluR1α were similar to values reported for rat mGluR1α in various expression systems (24, 29, 30). Interestingly, the EC_{50} value for glutamate-stimulated phosphoinositide hydrolysis in RGT/HmGluR1α cells was approximately 5-fold lower than values reported previously for glutamate-stimulated phosphoinositide hydrolysis in rat mGluR1α. This discrepancy is likely due to the efficient removal of glutamate from the extracellular medium by the rat GLAST transporter in the RGT/HmGluR1α cell line. In addition, rat mGluR1α receptor antagonists, such as MCPG and 4C3HPG (24, 25), inhibited HmGluR1α-mediated phosphoinositide hydrolysis in RGT/HmGluR1α cells. These results support the hypothesis that, in addition to their sequence homology, the rat and human mGluR1αs share a similar signal transduction pathway in cell lines and a similar pharmacological profile.

The lack of receptor coupling to the phosphoinositide hydrolysis pathway in AV12/HmGluR1α cells could be due to the absence of effective glutamate removal from the extracellular medium. Thus, we examined the effects of short- and long-term inhibition of glutamate uptake in RGT/HmGluR1α cells. We predicted that short-term exposure to the glutamate uptake blocker, *trans*-PDC, would result in a small, but significant, increase in glutamate in the extracellular medium. This would result in stimulation of HmGluR1α-mediated phosphoinositide hydrolysis (see Figs. 6A and 7A). HPLC analysis of glutamate levels in the medium revealed that the concentration of glutamate increased in medium to which *trans*-PDC was added. The concentration of glutamate achieved in the presence of 300 μM *trans*-PDC after incubation was similar to the concentration of glutamate added exogenously, suggesting that 300 μM *trans*-PDC greatly inhibits the rat GLAST transporter in RGT/HmGluR1α cells. [^{3}H]Glutamate uptake experiments confirmed this observation, where it was found that 300 μM *trans*-PDC blocked accumulation of glutamate by ~80%.

The acute addition of *trans*-PDC also led to an apparent increase in phosphoinositide hydrolysis. The present results suggest that in the case of *trans*-PDC, this apparent increase is likely due to uptake inhibition leading to glutamate activation of phosphoinositide hydrolysis, rather than direct activation of HmGluR1α by this compound. In contrast, the compounds 3,5-DHPG, 1*S*,3*R*-ACPD, quisqualate, and L-CCG-I appear to be direct-acting HmGluR1α agonists because they stimulate phosphoinositide hydrolysis at concentrations that have no effect on [^{3}H]glutamate uptake. These data demonstrate that the activation of phosphoinositide hydrolysis *per se* is not sufficient evidence that a compound is a direct-acting mGluR agonist.

Thomsen *et al.* (28) recently reported that a number of glutamate transporter inhibitors, including *trans*-PDC, stimulated phosphoinositide hydrolysis in baby hamster kidney cells expressing rat mGluR1α. As in the present study, they suggest that this is not due to direct activation of mGluR1α by uptake inhibitors; rather, it is an effect of increased glutamate levels in the extracellular medium. Thomsen *et al.* suggested that increased glutamate levels result from extrusion of glutamate into the medium through a heteroexchange with the uptake inhibitor/substrate. In the present study, we also found increased levels of glutamate in the extracellular medium in the presence of *trans*-PDC (see Fig. 6B). However, a similar concentration of glutamate is found in AV12/HmGluR1α cells, which do not express a glutamate transporter. Thus, in the present study, heteroexchange of glutamate with *trans*-PDC by the GLAST transporter cannot account for the increased glutamate concentration in the extracellular medium.

A decrease in agonist-stimulated phosphoinositide hydrolysis in RGT/HmGluR1α cells was observed on long-term inhibition of glutamate uptake, thus creating a decreased response similar to that seen in AV12/HmGluR1α cells. Examination of mRNA levels in both cell types revealed that decreased phosphoinositide hydrolysis in AV12/HmGluR1α cells was not due to the absence of expression of HmGluR1α mRNA. Thus, the lack of appreciable agonist-stimulated phosphoinositide hydrolysis in AV12/HmGluR1α cells and the inhibition of agonist-stimulated phosphoinositide hydrolysis in RGT/HmGluR1α cells on preexposure to *trans*-PDC are both likely due to desensitization of HmGluR1α. This phenomenon has been demonstrated previously for mGluR1α-mediated phosphoinositide hydrolysis. For example, preexposure of cultured cerebellar granule cells to glutamate has been reported to inhibit mGluR-mediated phosphoinositide hydrolysis (27). In addition, long-term application of mGluR1 agonists has been shown to result in decreased inositol-1,4,5-trisphosphate receptor protein levels (31) and decreased mGluR1 mRNA levels (32) in cerebellar granule cell cultures. Exposure of cultured cerebellar granule cells to high KCl concentrations has also been shown to inhibit mGluR-mediated phosphoinositide hydrolysis and to decease mGluR1α mRNA levels (33, 34). However, in high KCl, muscarinic receptor-mediated phosphoinositide hydrolysis was not inhibited (33), suggesting that KCl-induced depolarization elicits selective down-regulation of mGluR1α rather than a nonspecific down-regulation of all phosphoinositide hydrolysis-coupled receptors. Interestingly, high expression of mGluR1α has not been shown in granule cells but has been shown in Purkinje cells of the rat cerebellum (3, 35–37). These studies of phosphoinositide hydrolysis-linked mGluRs in nonrecombinant systems further exemplify the importance of the *in situ* environment in the regulation of mGluR expression and coupling.

In summary, we demonstrated that removal of glutamate from the extracellular medium plays an important role in the function of glutamate receptors expressed in cell lines. In the presence of the glutamate uptake inhibitor, *trans*-PDC, HmGluR1α-mediated phosphoinositide hydrolysis was enhanced as a result of short-term increases in extracellular glutamate. However, after exposure to *trans*-PDC for 24 hr, HmGluR1α-mediated phosphoinositide hydrolysis decreased in response to the prolonged increase in extracellular glutamate levels. The mRNA levels for HmGluR1α were not greatly different in AV12/HmGluR1α or in RGT/HmGluR1α, suggesting that the lack of AV12/HmGluR1α-mediated phos-

phoinositide hydrolysis could not be due to reduced expression of the receptor. These data indicate that HmGluR1α receptors undergo a profound desensitization on long-term preexposure to agonist. The mechanism by which HmGluR1α receptors desensitize is not yet known. Previous reports have shown that receptors coupled to the phosphoinositide hydrolysis pathway may undergo desensitization via a number of different mechanisms (38). Future studies will help to clarify the mechanism by which HmGluR1α receptors undergo desensitization. The cell line reported and characterized in this article, RGT/HmGluR1α, represents a well-controlled experimental system for further investigation of mGluR desensitization mechanisms.

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