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# Supersensitivity of Human Metabotropic Glutamate 1a Receptor Signaling in L929sA Cells

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## ABSTRACT

The effect of antagonist pretreatment on the signaling properties of the human metabotropic glutamate 1a (hmGlu1a) receptor was examined in stably transfected L929sA cells. Pre-exposure of hmGlu1a receptor-expressing cells to the mGlu1 receptor antagonists (*S*)-4-carboxy-3-hydroxyphenylglycine and 7-(hydroxyimino)cyclo-propa[*b*]chromen-1a-carboxylate ethyl ester dramatically enhanced subsequent glutamate-induced phosphoinositide hydrolysis and intracellular [$Ca^{2+}$] rise. We found clear indications that the antagonist-mediated enhancement of glutamate-evoked mGlu1a receptor signaling is caused by the development of mGlu1a receptor supersensitivity: the potency of glutamate was increased by 3-fold after 24 h antagonist pretreatment and the potency of the antagonists was significantly decreased in antagonist-pretreated cells. The kinetic profile of the antagonist-mediated enhancement showed that the maximal increase in intracellular [$Ca^{2+}$] was already reached after 30-min pretreatment, suggesting that de novo receptor synthesis is not involved in the process of mGlu1a receptor supersensitization. Glutamate-mediated phosphoinositide hydrolysis increased up to 24 h after antagonist treatment. Although it seemed likely that the hmGlu1a receptor could desensitize after activation by endogenously present glutamate, removal of glutamate from the extracellular medium with GPT resulted in a much smaller enhancement of glutamate responsiveness. Moreover, the magnitude of antagonist-mediated receptor supersensitivity was much larger than the magnitude of agonist-induced receptor desensitization. These results suggest that antagonist-evoked mGlu1 receptor supersensitivity is not merely the result of a blockade of agonist-induced desensitization. Finally, we found that antagonist pretreatment doubled the amount of receptors at the cell surface. Our findings are the first lines of evidence that prolonged antagonist treatment can supersensitize the hmGlu1a receptor. In view of the potential therapeutic application of mGlu1 receptor antagonists, it will be important to know whether these phenomena occur in vivo.

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So far, eight subtypes of the family of mGlu receptors have been cloned (mGlu1 to mGlu8 receptors). These receptors can be further subdivided into three groups based on their amino acid sequence homology, signal transduction mechanisms, and pharmacological profiles. Group I mGlu receptors consist of subtypes 1 and 5. Splice variants have been found for both the mGlu1 receptor (mGlu1a to mGlu1f receptor) and mGlu5 receptor (mGlu5a, mGlu5b, and mGlu5d receptors). Stimulation of group I mGlu receptors activates phosphoinositide hydrolysis, giving rise to the generation of the intracellular messengers diacylglycerol and inositol trisphosphate. This coupling in turn results in protein kinase C activation and $Ca^{2+}$ mobilization from the endoplasmic reticulum (Aramori and Nakanishi, 1992). Group II (mGlu2 and mGlu3 receptor) and group III mGlu receptors (mGlu4, -6, -7, and -8 receptors) both inhibit adenylate cyclase in heterologous expression systems (Pin and Duvoisin, 1995).

The mGlu1 receptor has been described as undergoing agonist-induced desensitization. This event seems to include a rapid component that may involve activation of protein kinase C (Catania et al., 1991; Thomsen et al., 1993; Alaluf et al., 1995; Ciruela et al., 1999a) and receptor internalization (Ciruela and McIlhinney, 1997; Doherty et al., 1999) and a slower, protein kinase C-independent component (Catania et al., 1991; Desai et al., 1996). Down-regulation of receptor

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**ABBREVIATIONS:** mGlu, metabotropic glutamate; (*S*)-4CPG, (*S*)-4-carboxyphenylglycine; (*S*)-4C3HPG, (*S*)-4-carboxy-3-hydroxyphenylglycine; IP, inositol phosphate; $IP_3$, inositol 1,4,5-trisphosphate; CPCCOEt, 7-(hydroxyimino)cyclo-propa[*b*]chromen-1a-carboxylate ethyl ester; rmIFN-$\beta$, recombinant mouse interferon $\beta$; GPT, glutamate pyruvate transaminase; AM, acetoxymethyl ester; IFN, interferon; HPLC, high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; PLC, phospholipase C; SIB 1893, 2-methyl-6-(2-phenylethenyl)pyridine.

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protein expression has been suggested to play a role in the latter process, because in cultured cerebellar granule cells, desensitization of the mGlu1 receptor has been shown to result in reduced levels of mGlu1 receptor mRNA upon exposure to glutamate or quisqualate or in the presence of high $K^+$ concentration (Favaron et al., 1992; Aronica et al., 1993; Bessho et al., 1993). Recently, a G protein-coupled receptor kinase-dependent mGlu1a receptor desensitization mechanism was described (Dale et al., 2000). Finally, attenuation of mGlu1 receptor signaling can occur at the level of the $IP_3$ receptor, because chronic mGlu1 receptor stimulation has been found to down-regulate $IP_3$ receptor expression in cerebellar granule cells (Simpson et al., 1994). Prézeau et al. (1996) showed that the presence of (*S*)-4CPG and (*S*)-4C3HPG in the culture medium resulted in a significant enhancement in glutamate-induced [$^3$H]IP production in LLC-PK1 cells expressing the mGlu1a receptor. When transfected BHK cells expressing the mGlu1a receptor were preexposed to (*R*,*S*)-1-aminoindan-1,5 dicarboxylic acid for 48 h, agonist-induced [$^3$H]IP formation was also increased (Moroni et al., 1997). It was presumed that this increase in the subsequent agonist-response was the result of a blockade of mGlu1 receptor desensitization induced by the presence of glutamate in the incubation medium. The effect of prolonged antagonist pretreatment on intrinsic receptor signaling properties was never studied.

However, it is known that long-term antagonist pretreatment can change the function and properties of a number of G protein-coupled receptors. For the dopamine receptor, for example, it has been shown that long-term treatment with dopamine receptor antagonists causes not only an enhancement of a subsequent agonist response but also an increase in agonist potency (Missale et al., 1989) and a decrease in antagonist potency (Burt et al., 1977). Importantly, this phenomenon, referred to as dopamine receptor supersensitivity, has been linked to the development of tardive dyskinesia, which is a serious long-term side effect of antipsychotic drugs (Crane, 1973; Baldessarini and Tarsy, 1980).

Excessive activation of group I mGlu receptors may participate in a variety of disorders of the central nervous system, such as pain, epilepsy, and ischemia (for review, see Bordi and Ugolini, 1999), and mGlu1 receptor antagonists may be of therapeutic use in a short- or long-term treatment schedule. Because antagonist-mediated changes in mGlu1 receptor signaling could thus be a concern in a clinical setting, we aimed our study at defining whether antagonist treatment would change subsequent signaling properties of the hmGlu1a receptor. We used an inducible expression system in L929sA cells, which confers the possibility of inducing hmGlu1a receptor expression when required. mGlu1 receptor signaling properties at the level of phosphoinositide hydrolysis and $Ca^{2+}$ mobilization were studied after pretreatment with CPCCOEt or (*S*)-4C3HPG. CPCCOEt has been described as the first mGlu1 receptor-specific allosteric noncompetitive antagonist (Annoura et al., 1996; Hermans et al., 1998; Litschig et al., 1999) and (*S*)-4C3HPG is known to antagonize mGlu1 receptors in a competitive manner (Thomsen et al., 1994; Brabet et al., 1995).

This article reports the first study of antagonist-mediated mGlu1a receptor supersensitivity. We identified the phenomenon of mGlu1a receptor supersensitivity and characterized several molecular events involved. We questioned whether supersensitivity is a result of blockade of agonist-induced desensitization and whether de novo receptor synthesis or a blockade of agonist-independent receptor activation is involved. Additionally, we evaluated the amount of cell surface mGlu1a receptors with and without antagonist pretreatment.

## Experimental Procedures

**Materials.** rmIFN-$\beta$ was generously provided by Dr. P. Vanhoenacker (University of Gent, Belgium). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). CPCCOEt and (*S*)-4C3HPG were both from Tocris Cookson (Essex, UK). Glutamate was from Aldrich Chemical Company (Milwaukee, WI). *myo*-[$^3$H]inositol was purchased from PerkinElmer Life Sciences (Boston, MA) and GPT from Roche Diagnostics GmbH (Mannheim, Germany). Fluo-3-AM and pluronic acid were from Molecular Probes (Leiden, The Netherlands). Both sulfo-NHS-SS-biotin and ImmunoPure Immobilized Streptavidin beads were from Pierce (Rockford, IL). The anti-mGlu1a receptor polyclonal antibody was from Chemicon International (Temecula, CA). Nonfat dry milk, Tween 20, and peroxidase-conjugated anti-rabbit IgG from donkey were all from Bio-Rad (Hercules, CA). Probenecid, pyruvate, *o*-phthalaldehyde, PMSF, Ponceau S, mercaptoethanol, and dantrolene were purchased from Sigma-Aldrich (Steinheim, Germany) and acetonitrile from Acros (Geel, Belgium). All other reagents were from Merck (Darmstadt, Germany).

**Cell Transfection and Culture.** hmGlu1a receptor cDNA was cloned into the pSP64 MxpA expression vector. In this vector, hmGlu1a receptor expression is under control of the murine Mx1 promoter (Hug et al., 1988), a 1600 base-pair fragment, which confers IFN-inducible expression on the insert (Vanhoenacker et al., 1997). L929sA cells, which contain an endogenous IFN receptor, were transfected with this hmGlu1a receptor-pSP64 MxpA construct and the selection vector pAG60 MT2 (Colbere-Garapin et al., 1981) using a modification of the calcium phosphate method as described previously (Lesage et al., 1998). A monoclonal cell line was isolated under geneticin-G418 selection (500 $\mu$g/ml) in GlutaMax I medium supplemented with 10% heat inactivated dialyzed fetal calf serum and antibiotics. The same medium was used for cell culturing. Geneticin-G418 was left out at least 1 day before assay. hmGlu1a receptor expression was induced by treatment of L929sA cells with 1000 U/ml rmIFN-$\beta$ for 24 h unless otherwise indicated.

**Primary Cerebellar Granule Cell Culture.** Primary cultures of cerebellar granule cells were prepared from 5- to 6-day-old Wistar rat pups. Cerebella were dissected, and cells were exposed to trypsin (0.05%, 15 min at 37°C) followed by addition of soybean trypsin inhibitor (0.28 mg/ml) and DNase I (0.05 mg/ml). Cells were then centrifuged and the pellet was resuspended and again triturated. After another centrifugation, the obtained pellet was resuspended in Basal Medium Eagle supplemented with 10% heat inactivated fetal calf serum, 25 mM KCl, and antibiotics. Cells were seeded at a density of $1.25 \times 10^6$ cells/well in 24-well plates (BD Labware, Le Pont De Claix, France) precoated with 10 $\mu$g/ml poly-L-lysine and maintained at 37°C at 95% air/5% $CO_2$ for 8 days; 10 $\mu$M cytosine $\beta$-D-arabinofuranoside was added after 4 days of culturing.

**IP Response in Primary Cerebellar Culture and hmGlu1 Receptor Expressing L929sA Cells.** Cerebellar neurons were cultured as described above and L929sA cells expressing the hmGlu1a receptor were seeded at 60,000 cells/well in 24-well plates (Becton Dickinson Labware, Le Pont De Claix, France) 48 h before the experiment. CPCCOEt and (*S*)-4C3HPG were added to the culture medium 24 h before the experiment unless otherwise stated, and cells were labeled with 2.5 $\mu$Ci/ml *myo*-[$^3$H]inositol (22 Ci/mmol) overnight. On the day of the experiment, cells were washed twice with controlled salt solution (25 mM Tris-HCl, 120 mM NaCl, 5.4 mM KCl, 0.3 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 15 mM glucose, pH 7.4

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at 37°C). After a 10-min preincubation in controlled salt solution containing 10 mM LiCl, agonist was added and the cells were incubated for 30 min at 37°C. Where GPT (3 U/ml) and pyruvate (2 mM) were added to decrease the glutamate concentration in the medium, cells were washed and incubated with these agents for a maximum of 8 h before the agonist challenge. Both compounds were repeatedly added each hour and finally washed out extensively. Where the effect of GPT per se was assessed, cells treated with GPT/pyruvate and with pyruvate only were compared. The formation of [$^3$H]IP was stopped by the addition of 100 $\mu$l of ice-cold 1 N $HClO_4$ to each well. Plates were placed at 4°C and incubates were neutralized to pH 7.4 by adding 100 $\mu$l of KOH/phosphate solution (0.5 M $K_2HPO_4$, adjusted to pH 7.5 with *o*-phosphoric acid and supplemented with KOH to a final concentration of 1 M, pH 13.0). After 30 min, proteins were removed by centrifugation (2000*g* for 5 min) and the samples were applied to ion exchange chromatography, Dowex AGI-X8 (Bio-Rad) columns. [$^3$H]Inositol 1-phosphate, [$^3$H]inositol 1,4-bisphosphate, and [$^3$H]$IP_3$ were eluted with 3.5 ml of 0.1 M formic acid/1 M ammonium formate (pH 4.1) and counted in a Packard liquid scintillation counter with Ultima Flo AF (Packard, Groningen, The Netherlands) as scintillant fluid. In each experiment, concentration curves were run in triplicate.

**Intracellular Calcium Response in hmGlu1 Receptor Expressing Cells.** Intracellular calcium ion levels ($[Ca^{2+}]_i$) were measured with the fluorescent indicator dye fluo-3-AM using a fluorometric imaging plate reader (Molecular Devices, Menlo Park, CA). L929sA cells expressing the hmGlu1a receptor were seeded at 25,000 cells/well in 96-well black/clear bottom plates (COSTAR, Cambridge, MA) 24 h before the experiment. CPCCOEt or (*S*)-4C3HPG was added to the medium 24 h before loading unless otherwise stated. The day of the experiment, the cells were loaded for 1 h with 2 $\mu$M fluo-3-AM in culture medium at 37°C in 95% air/5% $CO_2$. Fluo-3-AM was dissolved in 20% pluronic acid/dimethyl sulfoxide to facilitate loading of the dye into the cells. During the loading time, 5 mM probenecid, which inhibits $P_{50}$-glycoprotein mediated transport of the dye out of the cell, was present, and the pH of the medium was adjusted to 7.4 with 1 M HEPES. The cells were washed twice with 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM glucose, 1.25 mM $CaCl_2$, 5 mM HEPES, and 2.5 mM probenecid, pH 7.4, and were preincubated for 20 min at 37°C with antagonist or buffer before measurement. The effect of GPT was assessed as in the IP assay. Relative fluorescent units were recorded for each well in function of time. The fluorescent peak obtained upon the addition of glutamate was taken as the signal. In each experiment, concentration response curves were run in triplicate or quadruplicate.

**HPLC Analysis of Glutamate Concentrations.** In experiments where GPT was involved, HPLC analysis was used to determine the levels of glutamate in the extracellular medium. L929sA cells expressing the hmGlu1a receptor were seeded as described for measurements of IP accumulation or $Ca^{2+}$ mobilization. For the glutamate assay, medium was added instead of *myo*-[$^3$H]inositol. Medium or buffer (1 ml) was collected at different time-points in the experiment and was diluted with HCl to a final concentration of 0.01 N HCl. The samples were left at room temperature for 10 min while shaking them regularly; after 10 min of centrifugation at 2,000*g*, the supernatant was filtered through a 0.22-$\mu$m filter mesh and kept at −20°C until analysis. At the day of HPLC analysis, the samples were thawed and centrifuged for 1 min at 15,000*g*. Precolumn derivatization was carried out with a Varian 9100 Autosampler (Varian, Palo Alto, CA), operating at 4°C. The samples were mixed for 2 min with equal volumes of *o*-phthalaldehyde (200 $\mu$g/ml in 100 mM borate buffer, pH 10.4, containing 0.4 $\mu$l/ml mercaptoethanol). The mixture (20 $\mu$l) was subsequently injected on a Hypersyl BDS $C_{18}$ column (particle diameter, 3 $\mu$m; 4.6 × 100 mm) at 40°C. The HPLC system consisted of a Varian 9010 Liquid Chromatograph equipped with a fluorescence detector (excitation/emission wavelengths, 330/450 nm; FP920; Jasco, Tokyo, Japan). The mobile phase consisted of 50 mM $K_3PO_4$, pH 7.0, containing 7% acetonitrile (A) and 60% $H_2O$-40% acetonitrile (B). Gradient elution was performed at 1 ml/min. Initial conditions (95% A/5% B) were changed over 5 min to 10% B, followed by an increase to 100% B in 1 min. This condition was maintained for 3 min before returning to the initial conditions. Retention time for glutamate was 6 min. For quantitation, standard curves were prepared by *o*-phthalaldehyde derivatization and analysis of known amounts of glutamate (ranging from 0.05 to 2 $\mu$M).

**Measurement of Inositol Phospholipid Labeling.** When inositol phospholipid labeling was assessed, cell monolayers were extracted with a mixture of chloroform/methanol/10 M HCl [100:200:1 (v/v)] after aspiration of the medium. Chloroform and water were then added and, after centrifugation, the lower chloroform phase was dried at room temperature and radioactivity was determined.

**Crude Membrane Preparation, Biotinylation of Cell Surface Proteins, and Western Blot.** For crude membrane preparations, hmGlu1a receptor expressing L929sA cells were grown in 145-mm plates until 80% confluence and were then induced with 1000 U/ml rmIFN-$\beta$ for 24 h. Cells were washed twice with PBS and stored at −70°C until membrane preparation. After thawing, cells were suspended in 50 mM Tris-HCl, pH 7.4, supplemented with 1 mM PMSF (a proteolytic enzyme inhibitor) and centrifuged for 10 min at 23,500*g*. The supernatant was decanted and the pellet resuspended in 10 mM Tris-HCl pH 7.4, 1 mM PMSF. After recentrifugation for 20 min at 30,000*g*, the pellet was homogenized in 50 mM Tris-HCl, pH 7.4. Protein concentrations were measured by the Bio-Rad protein assay with bovine serum albumin as standard. Membrane protein (20 $\mu$g) was used for immunoblotting.

Cell surface proteins were biotinylated as follows. hmGlu1a receptor expressing L929sA cells were washed twice with PBS and then incubated with 0.5 mg/ml sulfo-NHS-SS-biotin in PBS for 30 min at

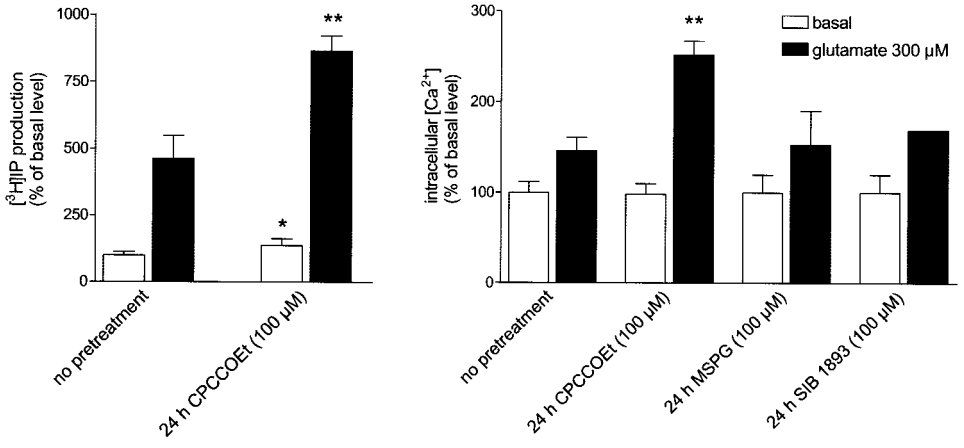


**Fig. 1.** Glutamate-induced [$^3$H]IP formation and $Ca^{2+}$-mobilization in L929sA cells expressing the hmGlu1a receptor. Cells were stimulated with 300 $\mu$M glutamate. Values are expressed as percentage of the signal obtained when cells are treated with buffer (basal level: 4615 ± 602 d.p.m. for [$^3$H]IP measurements; 9728 ± 901 relative fluorescent units for $Ca^{2+}$ measurements) and are mean ± S.D. of four to five individual experiments. Statistical analysis was performed using the Student's *t* test (two-tailed). Values statistically different from the buffer treated glutamate response are indicated: ⋆ $p < 0.05$; ⋆⋆ $p < 0.01$.

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4°C. The remaining biotin was then quenched by incubating the cells with 100 mM glycine for 15 min. Cells were washed twice with PBS and solubilized with ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EGTA, 1 mM EDTA, and 0.1 mM PMSF, pH 7.4) for 30 min. After solubilization, the cell lysate was collected and centrifuged for 15 min at 30,000*g*. The supernatant was then incubated with ImmunoPure Immobilized Streptavidin beads for 2 h with constant rotation at 4°C. The beads were collected by brief centrifugation and were washed twice with lysis buffer and once with PBS. Immune complexes were dissociated by adding 150 $\mu$l of SDS sample buffer and 100 mM DTT and heating to 100°C for 5 min. Samples were centrifuged and 30 $\mu$l of the supernatant was immunoblotted as described below.

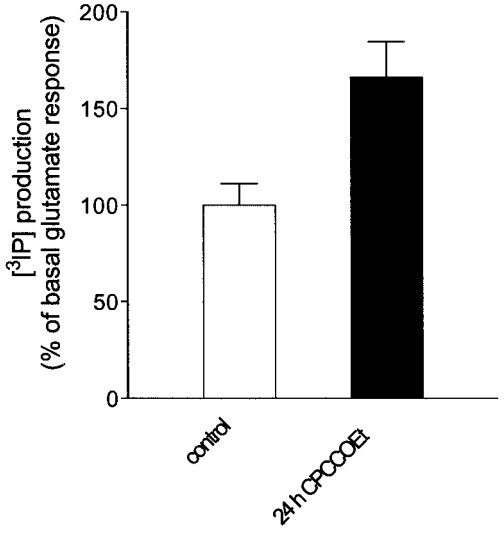


**Fig. 2.** Glutamate-induced [$^3$H]IP formation in cultured cerebellar neurons. Cells were stimulated with 1 mM glutamate. Values are expressed as percentage of the signal seen for 1 mM glutamate in the absence of antagonist treatment and are mean ± S.D. of two individual experiments performed in triplicate.

For immunoblotting, membrane protein was subjected to SDS-polyacrylamide gel electrophoresis (using a 7.5% Tris-HCl gel; Bio-Rad) and transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) by electroblotting. To ensure that equivalent amounts of protein were loaded in each lane and that transfer was comparable, membranes were stained with Ponceau S before immunoblotting. Blots were blocked overnight with 5% nonfat dry milk/0.1% Tween 20 in PBS and incubated for 1 h with the first and secondary antibody. The primary antibody (affinity purified polyclonal antibody against a C-terminal sequence of the mGlu1a receptor) was diluted 1:200 in PBS containing 2.5% nonfat dry milk; the secondary antibody (peroxidase-conjugated anti-rabbit IgG from donkey) was diluted 1:5000. Detection was performed by using the chemiluminescence plus (ECL+) Western blotting analysis system (Amersham Biosciences). Densitometric analysis was performed using the MCID-M4 imaging system.

**Data Analysis and Statistics.** Concentration response data were analyzed using nonlinear regression analysis (Prism; GraphPad, San Diego, CA) by fitting the equation: $E = E_{max}/(1 + EC_{50}/C)^n$, where E is the measured response at a given agonist concentration (C), $E_{max}$ is the maximal response, $EC_{50}$ is the concentration producing 50% stimulation, and $n$ is the slope index.

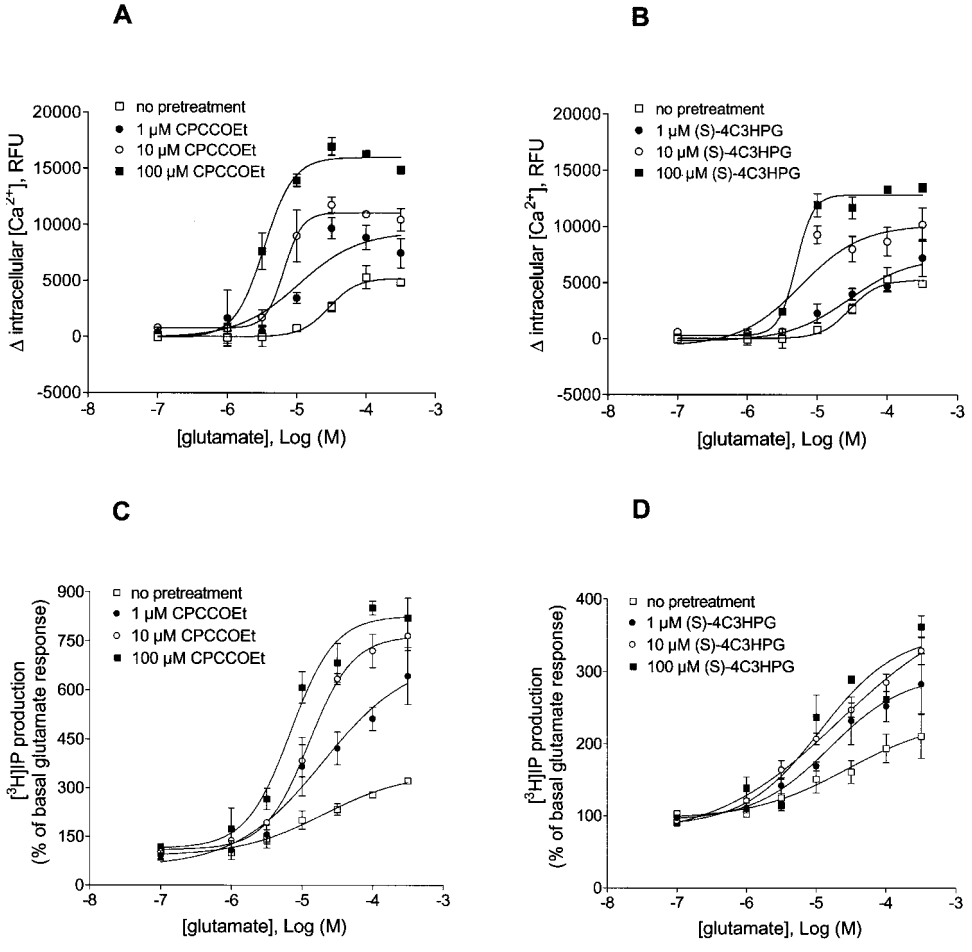


**Fig. 3.** Effect of preincubation of L929sA cells expressing the hmGlu1a receptor with increasing concentrations of CPCCOEt or (*S*)-4C3HPG on glutamate-induced signaling. A, B, effect of increasing concentrations of CPCCOEt or (*S*)-4C3HPG on glutamate-induced $Ca^{2+}$ mobilization. C, D, effect of increasing concentrations of CPCCOEt or (*S*)-4C3HPG on glutamate-induced [$^3$H]IP accumulation. For the graphs shown in A and B values are mean ± S.D. of triplicate determinations within one experiment. Two additional experiments showed similar results. For the graphs shown in C and D, the response to glutamate is expressed as percentage of the signal obtained when cells were treated with buffer. Values are mean ± S.D. of three independent experiments performed in triplicate.

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Calculation of apparent equilibrium inhibition constants ($K_b$) was determined with the equation: $K_b = IC_{50}\,[1 + (C)\,(EC_{50})^{-1}]^{-1}$, where $IC_{50}$ is the antagonist concentration producing 50% inhibition at a given agonist concentration (C) and $EC_{50}$ is the agonist concentration producing 50% stimulation. The two-tailed Student's $t$ test was used for statistical evaluation of the data.

## Results

**Effect of mGlu1a Receptor Antagonist Pretreatment on Glutamate-Induced mGlu1a Receptor Signaling.** Glutamate-induced phosphoinositide hydrolysis and $Ca^{2+}$ mobilization in L929sA cells expressing the hmGlu1a receptor with and without pretreatment with the mGlu1 receptor antagonist CPCCOEt are shown in Fig. 1. Pretreatment of the cells with CPCCOEt for 24 h resulted in a 2-fold enhancement of glutamate-induced [$^3$H]IP production and intracellular [$Ca^{2+}$] rise. The fact that the glutamate response increased after washout of the noncompetitive compound CPCCOEt indicates that its antagonist action on hmGlu1a receptor is reversible. Basal [$^3$H]IP formation was slightly yet significantly increased (26%), whereas basal intracellular [$Ca^{2+}$] was not altered when cells were pretreated with CPCCOEt. Pre-exposure to (*R*,*S*)-α-methyl-4-sulfonophenylglycine or SIB 1893, which act as selective antagonists toward the mGlu2 and -5 receptor, respectively, did not increase glutamate-induced $Ca^{2+}$ mobilization, demonstrating the specific effect of mGlu1 receptor antagonists on the mGlu1a receptor-mediated glutamate response. Importantly, preliminary data show that the glutamate-mediated IP response is also increased to 166% in cultured cerebellar neurons ($n = 2$) upon 24-h CPCCOEt treatment (Fig. 2).

TABLE 1

The potency ($pEC_{50}$) of glutamate for $Ca^{2+}$ mobilization and [$^3$H]IP formation in L929sA cells expressing the hmGlu1a receptor after preincubation of the cells with increasing concentrations of CPCCOEt or (S)-4C3HPG

Values shown are mean ± S.D. of three experiments. Statistical analysis was performed using the Student's $t$ test (two-tailed).

| Antagonist Concentration | Glutamate ($pEC_{50}$) | |
|---|---|---|
| | $Ca^{2+}$ Mobilization | [$^3$H]IP Formation |
| CPCCOEt | | |
| 0 | 4.8 ± 0.2 | 4.6 ± 0.2 |
| 1 μM | 5.1 ± 0.2 | 4.5 ± 0.5 |
| 10 μM | 5.3 ± 0.1* | 4.9 ± 0.1 |
| 100 μM | 5.4 ± 0.1** | 5.1 ± 0.2* |
| (*S*)-4C3HPG | | |
| 0 | 4.8 ± 0.2 | 4.8 ± 0.3 |
| 1 μM | 5.1 ± 0.1 | 4.9 ± 0.3 |
| 10 μM | 5.2 ± 0.1* | 4.8 ± 0.5 |
| 100 μM | 5.2 ± 0.1* | 5.2 ± 0.2* |

* $p < 0.05$; ** $p < 0.01$.

**Effect of Antagonist Pretreatment on Agonist and Antagonist Potency of the hmGlu1a Receptor.** With a view to therapeutic applications, it was of considerable interest to know whether antagonist treatment affected the pharmacological properties of the hmGlu1a receptor. Therefore, we first investigated the potency of glutamate in untreated and antagonist-pretreated hmGlu1a receptor expressing L929sA cells. We determined the $EC_{50}$ values for glutamate after pretreatment with increasing concentrations of either CPCCOEt or (*S*)-4C3HPG, a competitive mGlu1a receptor antagonist, in a $Ca^{2+}$ mobilization assay (Fig. 3, A and B) and at IP level (Fig. 3, C and D). Figure 3 shows clearly that pretreatment of cells with both the noncompetitive and competitive antagonist increases subsequent glutamate-induced $Ca^{2+}$ and IP signaling. At the $Ca^{2+}$ level (Fig. 3, A and B; Table 1), the response to glutamate increased dose dependently and the maximal stimulation of the glutamate signal was about 2-fold for both antagonists. Furthermore, the glutamate potency for activating hmGlu1a receptor signaling was significantly lower in untreated versus antagonist pretreated cells (Table 1). At the IP level (Fig. 3, C and D; Table 1), pre-exposure of the cells to the mGlu1 receptor antagonists increased glutamate-mediated [$^3$H]IP formation to about 200% and glutamate potency was again significantly increased by 3-fold when the cells were pretreated with 100 μM CPCCOEt or 100 μM (*S*)-4C3HPG. To investigate whether the potentiating effect was also observed with other mGlu1 receptor agonists, we performed concentration-response curves of quisqualate. Pretreatment with 100 μM CPCCOEt or (*S*)-4C3HPG increased the maximal quisqualate response to about 150% and again significantly enhanced quisqualate $pEC_{50}$ from 5.4 to 6.0 for both antagonists ($n = 3$; $p < 0.01$).

Second, it was important to know whether the ability of the antagonists to block the agonist-induced response would be changed when the receptor was pre-exposed to the agent. We thus investigated antagonist potency on the naïve receptor and compared it with the antagonist potency after the receptor had been pre-exposed to the corresponding antagonist for 24 h (Fig. 4). The $K_b$ values of CPCCOEt and (*S*)-4C3HPG for inhibition of the glutamate-induced $Ca^{2+}$ mobilization with and without pre-exposure to antagonist are calculated from the respective $IC_{50}$ values and are shown in Table 2. The data illustrate that CPCCOEt and (*S*)-4C3HPG exhibit a significant 2- to 3-fold lower potency in antagonist-pretreated cells.

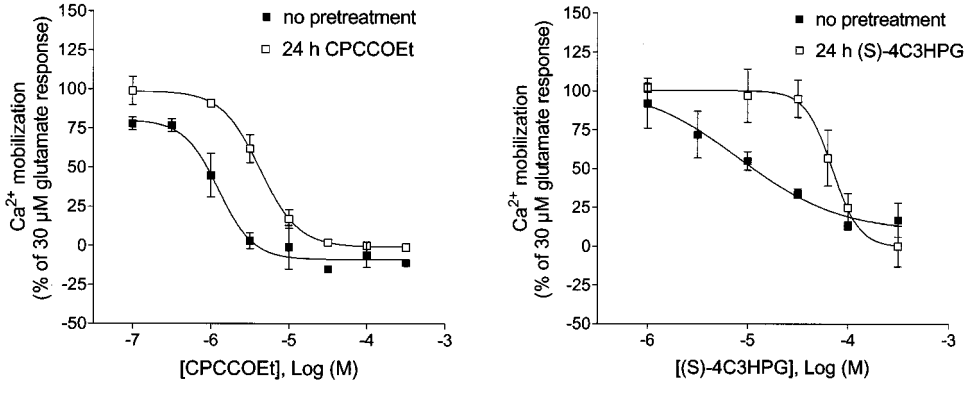


**Fig. 4.** Effect of antagonist pretreatment on blockade of glutamate-induced $Ca^{2+}$ mobilization at the hmGlu1a receptor expressed in L929sA cells. Cells were stimulated with 30 μM glutamate and were pretreated with antagonist [100 μM CPCCOEt or 300 μM (*S*)-4C3HPG] for 24 h or not. Values are expressed as percentage of the glutamate (30 μM)-stimulated $Ca^{2+}$ mobilization, which was set at 100%. Values are mean ± S.D. of three experiments.

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Alternatively, we examined whether treatment with one of the antagonists could change the potency of the other. As can be seen in Table 2, also in this situation higher concentrations of antagonist are needed to inhibit receptor activation by the same glutamate concentration.

**Kinetics of the Antagonist-Mediated Augmentation of the Glutamate Signal.** To shed light on the molecular events involved in mGlu1 receptor supersensitivity, we investigated the kinetics with which this phenomenon develops. For this, we examined the pretreatment time needed to increase the glutamate response. Figure 5 shows the effect of both CPCCOEt and (*S*)-4C3HPG on glutamate-induced $Ca^{2+}$ mobilization (Fig. 5A) and [$^3$H]IP production (Fig. 5B) in function of pretreatment time. Exposure of the cells to CPCCOEt or (*S*)-4C3HPG for 30 min is sufficient to maximally enhance the glutamate-induced intracellular $Ca^{2+}$ level about 2-fold (Fig. 5A). This level is maintained at least up to 24 h after pretreatment (i.e., there is no further rise in intracellular [$Ca^{2+}$] from 30 min pretreatment on). The plateau found when measuring intracellular [$Ca^{2+}$] was not caused by saturation of fluo-3-AM because the maximal $Ca^{2+}$ fluorescence signal, obtained by application of ionomycin, was consistently about 2.3-fold higher compared with addition of 300 $\mu$M glutamate after 30 min antagonist pretreatment (data not shown). In contrast to the fast enhancement of the signal seen at the level of intracellular [$Ca^{2+}$], glutamate-induced [$^3$H]IP production showed a slow and gradual increase up to 24 h pretreatment with either CPCCOEt or (*S*)-4C3HPG (Fig. 5B). The glutamate signal was again maximally augmented to about 200%. Different exposure times to antagonist had no effect on the amount of label incorporated into the phospholipid pool of the membrane, as can be seen from Table 3. However, during the 30-min glutamate treatment that is used to accumulate IP products, the administered glutamate (300 $\mu$M) may activate and subsequently desensitize a certain amount of receptors. Such assay-inherent desensitization would lead to underestimated amounts of IP. Therefore, in an attempt to prevent the potential glutamate-induced receptor desensitization, we also investigated the time course of antagonist-mediated enhancement of IP production after only 5 min glutamate treatment time (Fig. 6). Although the glutamate response now increased significantly after as little as 30 min CPCCOEt pretreatment, a further enhancement in IP signaling could still be seen up to 6 h before incubation.

**Effect of GPT Treatment on Glutamate-Induced hmGlu1a Receptor Signaling.** Another relevant question was whether antagonist-evoked mGlu1 receptor supersensitivity was caused by a blockade of mGlu1 receptor desensitization mediated by endogenous glutamate in the medium.

TABLE 2

Potencies of the antagonists to block glutamate-induced $Ca^{2+}$ signaling in a classic antagonist experiment ($K_b$ values)

For inhibition experiments, cells were either preincubated for 24 h with 100 $\mu$M CPCCOEt (24-h CPCCOEt), 300 $\mu$M (*S*)-4C3HPG [24 h (S)-4C3HPG], or not (no pretreatment). Values are mean ± S.D. of three to five experiments. Statistical analysis was performed using the Student's *t*-test (two-tailed).

| | $K_b$ | | |
|---|---|---|---|
| | No Pretreatment | 24-h CPCCOEt | 24-h (*S*)-4C3HPG |
| | | $\mu M$ | |
| CPCCOEt | 0.77 ± 0.24 | 1.24 ± 0.20* | 1.87 ± 0.14** |
| (*S*)-4C3HPG | 7.04 ± 1.49 | 19.47 ± 0.65*** | 24.30 ± 3.39*** |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

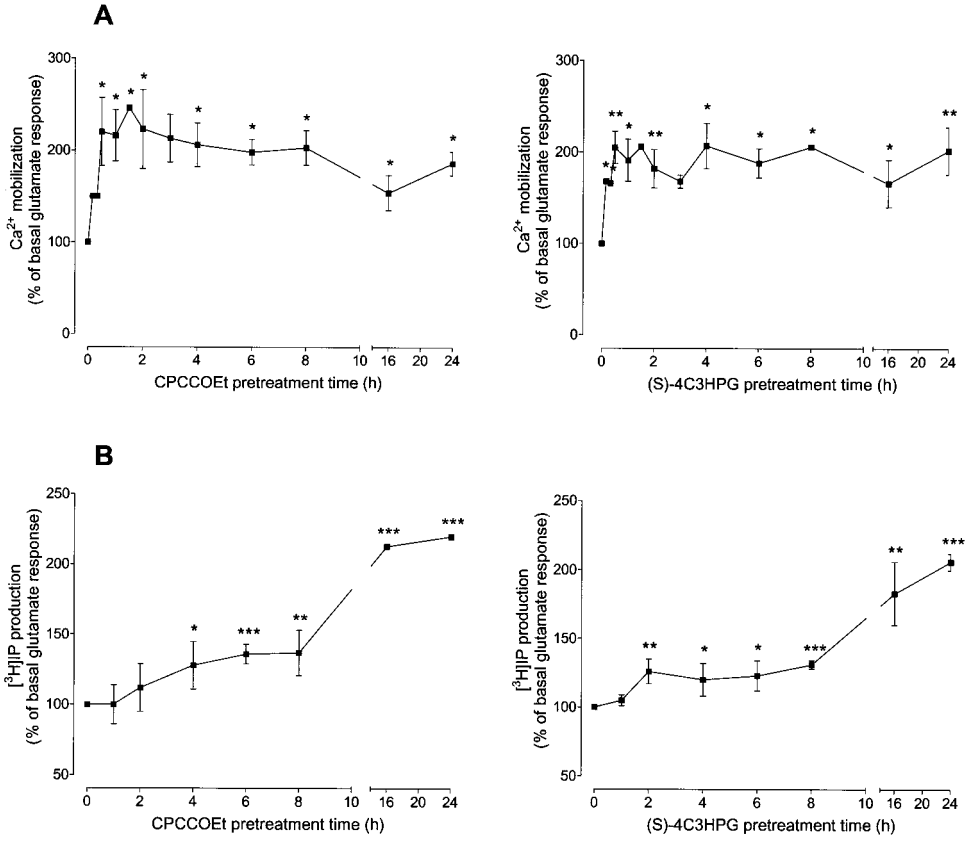


**Fig. 5.** Time course of antagonist-mediated enhancement of glutamate-induced signaling in L929sA cells expressing the hmGlu1a receptor. A, effect of CPCCOEt (100 $\mu$M) and (*S*)-4C3HPG (300 $\mu$M) pretreatment on glutamate-induced $Ca^{2+}$ mobilization. B, effect of CPCCOEt (100 $\mu$M) and (*S*)-4C3HPG (300 $\mu$M) pretreatment on glutamate-induced [$^3$H]IP accumulation. Cells were treated for different times with antagonist, the compound was washed-out, and cells were stimulated with 300 $\mu$M glutamate. Values are expressed as percentage of the signal seen in the 'control' response, with 300 $\mu$M glutamate and no antagonist pretreatment. Values are mean ± S.D. of three experiments. Values statistically different from the control glutamate response are indicated: ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$; ⋆⋆⋆, $p < 0.001$ using the Student's *t* test (two-tailed).

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As can be seen from Table 4, the extracellular glutamate concentration increases fast after refreshing the medium in regular $Ca^{2+}$ or IP experiments (no GPT added). Glutamate levels of 30 to 50 $\mu$M are already reached within 4 h. In a typical experiment, antagonists were added to the culture medium for the last 24 h before $Ca^{2+}$ or IP experiments. After this preincubation period, glutamate levels in the extracellular medium were accumulated up to 101 ± 24 $\mu$M ($n$ = 6). To check whether the mGlu1 receptor antagonists blocked activation of the mGlu1 receptor at this endogenous concentration, we performed a dose-response curve of glutamate in the presence of 100 $\mu$M CPCCOEt or 300 $\mu$M ($S$)-4C3HPG. Figure 7 shows that CPCCOEt fully prevents activation of the receptor up to 1 mM glutamate, consistent with a noncompetitive action. In contrast, ($S$)-4C3HPG did not fully block signaling by 100 $\mu$M glutamate or more and caused a parallel shift to the right of the concentration-response curve in line with a competitive inhibition. Thus, with glutamate levels being at maximum about 100 $\mu$M during antagonist preincubation, CPCCOEt would be able to block agonist-evoked desensitization. Because 300 $\mu$M ($S$)-4C3HPG is able to block only 80% of a 100 $\mu$M effect of glutamate, it is possible that this compound still allows some receptor activation and desensitization.

We then examined whether decreasing the concentration of endogenous glutamate in the medium would enhance the glutamate responses at IP and $Ca^{2+}$ level with the same time course and to the same extent as the antagonists. For this, we investigated the effect of medium refreshment with or without GPT addition on glutamate signaling in function of time (Fig. 8). With repeated addition of GPT (1 addition per hour), glutamate levels are kept below 2 $\mu$M up to 2 h. Glutamate-induced [$^3$H]IP production showed a small (35%) but nonsignificant increase at 30 min after a single medium switch and no GPT addition (Fig. 8A). The decrease in response observed thereafter is accompanied by a robust increase in extracellular glutamate levels, which is consistent with a loss of signal through glutamate-induced desensitization of the receptor. A medium shift plus a repeated addition of GPT increased glutamate-induced IP signaling to the same extent; it peaked at 1 h (28%). Even though glutamate levels were kept 5- to 15-fold lower than in normal medium, glutamate-induced IP formation declined thereafter. A similar picture was found when glutamate-induced $Ca^{2+}$ mobilization was investigated (Fig. 8B). In this case, the response at 30 min after the medium shift was increased by about 25%, whereas glutamate-induced desensitization of hmGlu1a receptor was less pronounced. These data show that mGlu1 receptor signaling under GPT conditions is not different from the signaling seen early after a medium shift, both at the IP and $Ca^{2+}$ levels. Remarkably, the 30-min GPT incubation was not at all able to enhance glutamate-induced $Ca^{2+}$ mobilization to the same extent as antagonist pretreatment (Fig. 5A), even though extracellular glutamate concentrations were in the submicromolar range (i.e., below glutamate levels that can stimulate the receptor) (Fig. 7).

**Constitutive hmGlu1a Receptor Signaling in rmIFN-$\beta$–Induced L929sA Cells.** Because it has been shown that pretreatment with inverse agonists can cause subsequent enhanced responsiveness of constitutively active G protein-coupled receptors (MacEwan and Milligan, 1996; Leurs et al., 1998; Stevens et al., 2000), we tested whether CPCCOEt or ($S$)-4C3HPG could act as inverse agonists. Therefore, we first examined basal hmGlu1a receptor activity in rmIFN-$\beta$–induced and noninduced cells. rmIFN-$\beta$–induced cells show

TABLE 3

Total phospholipid labeling after different incubation times with the mGlu1 receptor antagonist CPCCOEt

Values are expressed as a percentage of total *myo*-[$^3$H]inositol added and are mean ± S.D. of two experiments.

| CPCCOEt Incubation Time | Total Phospholipid Labeling |
|---|---|
| *h* | % |
| 0 | 1.4 ± 0.2 |
| 1 | 1.3 ± 0.1 |
| 2 | 1.4 ± 0.3 |
| 4 | 1.2 ± 0.0 |
| 6 | 1.4 ± 0.4 |
| 8 | 1.4 ± 0.4 |
| 24 | 1.2 ± 0.4 |

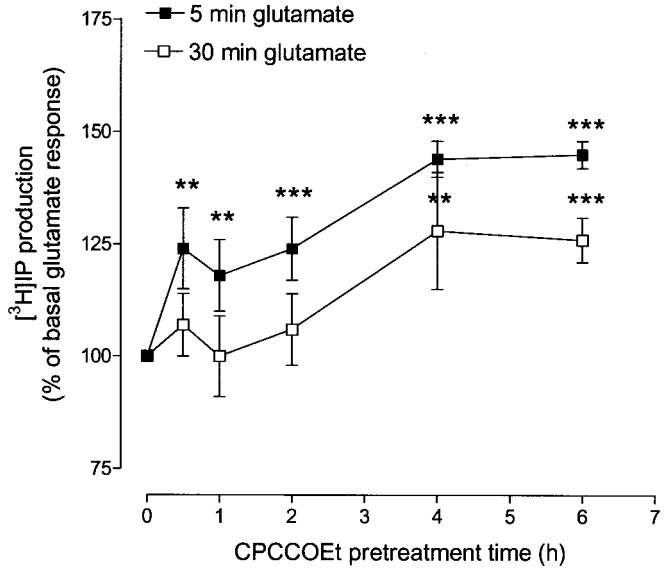


**Fig. 6.** Effect of decreasing glutamate treatment on time course of antagonist-mediated enhancement of IP accumulation. [$^3$H]IP formation was measured using 5- or 30-min glutamate (300 $\mu$M) treatment after various preincubation times with CPCCOEt (100 $\mu$M). Values are expressed as percentage of the signal seen for 300 $\mu$M glutamate in the absence of antagonist treatment. Values are mean ± S.D. of three experiments. Values statistically different from the buffer treated glutamate response are indicated: ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$; ⋆⋆⋆, $p < 0.001$ using the Student's $t$ test (two-tailed).

TABLE 4

Levels of endogenous glutamate in the medium for IP or $Ca^{2+}$ assays

Glutamate concentrations were measured at different times after medium refreshment without or with repeated addition (i.e., repeatedly added each hour) of GPT (3 U/ml) and pyruvate (2 mM). Values are from one representative experiment.

| Time After Medium Refreshment | Glutamate Concentration | | | |
|---|---|---|---|---|
| | IP Assay | | $Ca^{2+}$ Assay | |
| | Without GPT | With GPT | Without GPT | With GPT |
| *h* | *μM* | | | |
| 0.5 | 0.674 | 0.28 | 0.684 | 0.15 |
| 1 | 1.254 | 0.27 | 1.093 | 0.17 |
| 2 | 14.94 | 1.51 | 12.93 | 1.72 |
| 4 | 52.0 | 6.02 | 29.6 | 7.99 |
| 6 | 82.0 | 11.93 | 45.1 | 7.34 |
| 8 | 76.8 | 14.06 | 53.1 | 8.42 |

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high receptor levels whereas in noninduced cells some leakage of the promoter results in weak receptor expression, as illustrated in a Western blot using an antibody against the mGlu1a receptor (Fig. 9). Figure 10A shows that cells with high receptor expression have a higher level of basal IP production compared with cells with low receptor expression, reflecting that the hmGlu1a receptor can serve as a constitutively active receptor system in L929sA cells. In noninduced cells, the compounds did not exert inverse agonist activity (data not shown). Incubation (30-min) with maximal concentrations of CPCCOEt or (*S*)-4C3HPG consistently reduced basal IP accumulation in rmIFN-$\beta$–induced cells to the level seen at low receptor expression (Fig. 10B). Because glutamate concentrations in the medium during the 30-min incubation are too low to activate the receptor (Table 4 and Fig. 7), this effect might point toward inverse agonist activities of both compounds.

**Effect of Antagonist Pretreatment on mGlu1a Receptor Cell Surface Expression.** To examine whether 24-h antagonist treatment affected mGlu1a receptor expression at the plasma membrane, we biotinylated cell surface proteins using a membrane impermeant biotin ester. mGlu1a receptors specifically expressed at the plasma membrane were then detected by immunoblotting. After this procedure we clearly demonstrate an enrichment of the amount of mGlu1a receptors at the cell surface after 24 h CPCCOEt pretreatment (Fig. 11A). Densitometric analysis of cell surface immunoreactivity running at about 142 and 280 kDa, corresponding to the monomeric and dimeric mGlu1a receptor form, respectively, revealed that the amount of monomeric mGlu1a receptors increased to 326 ± 76% ($n = 3$, $p < 0.01$) and the amount of dimeric receptor to 263 ± 64% ($n = 3$, $p < 0.05$). The total amount of mGlu1a receptors, detected by immunoblotting of crude membrane preparations of hmGlu1a receptor expressing L929sA cells, was not affected (Fig. 11B).

## Discussion

**Treatment with mGlu1 Receptor Antagonists: Pharmacological Changes of the mGlu1a Receptor.** In line with previous reports (Prézeau et al., 1996; Moroni et al., 1997), our data show that antagonist pretreatment of the hmGlu1a receptor expressed in L929sA cells causes enhanced [$^3$H]IP production. We found that the glutamate-induced increase in intracellular $Ca^{2+}$ level was also potentiated after antagonist exposure and that reducing extracellular glutamate concentrations with GPT was less effective. The increases could be obtained with a noncompetitive (CPCCOEt) as well as a competitive [(*S*)-4C3HPG] mGlu1 receptor antagonist but not with non-mGlu1 receptor antagonists.

We found clear indications that the enhancement of the glutamate-evoked mGlu1a receptor response after 24-h antagonist pretreatment was caused by the development of mGlu1a receptor supersensitivity. Glutamate concentration-response curves (Fig. 3 and Table 1) showed that the glutamate potency was increased when measuring IP or $Ca^{2+}$

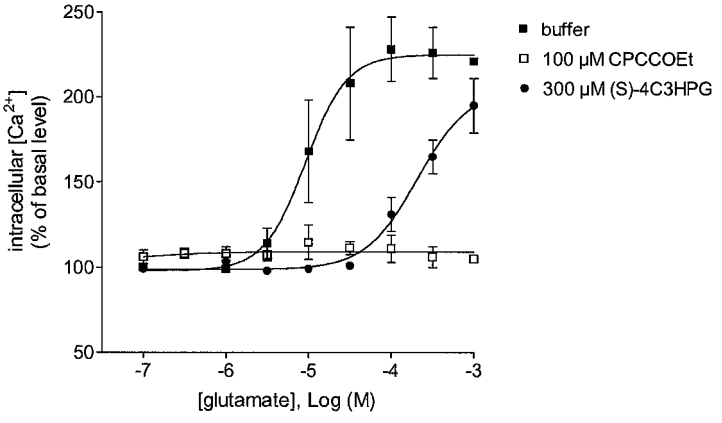


**Fig. 7.** Concentration-response curve of glutamate-induced $Ca^{2+}$ mobilization of the hmGlu1a receptor expressed in L929sA cells. Shown is a concentration-response curve of glutamate-induced rise in intracellular [$Ca^{2+}$] and antagonism thereof by 100 $\mu$M CPCCOEt and 300 $\mu$M (*S*)-4C3HPG. Values are expressed as percentage of basal intracellular [$Ca^{2+}$] levels and are mean ± S.D. of three experiments.

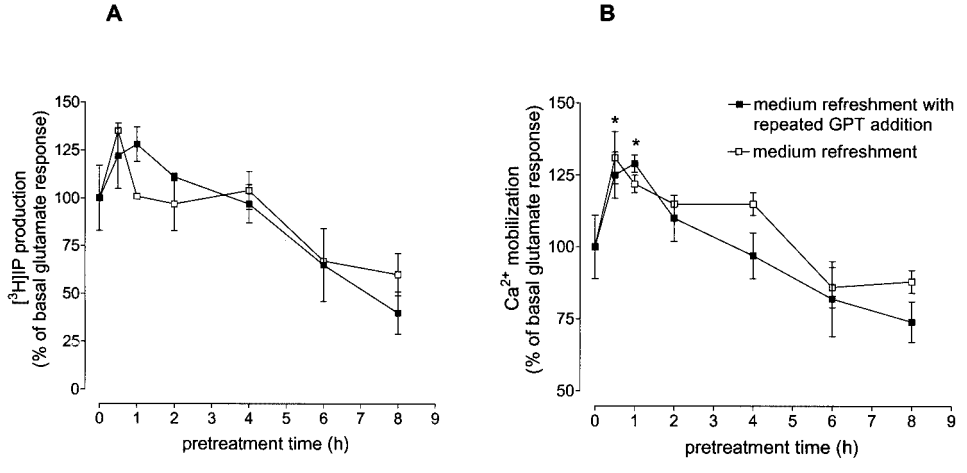


**Fig. 8.** Effect of medium refreshment and medium refreshment accompanied with repeated GPT (3 U/ml) and pyruvate (2 mM) addition to the medium on glutamate-induced [$^3$H]IP formation (A) and intracellular $Ca^{2+}$ release (B) in L929sA cells expressing the hmGlu1a receptor. Responses were followed over time after medium refreshment. Values are expressed as percentage of the signal obtained for 300 $\mu$M glutamate in the absence of GPT pretreatment. Values are mean ± S.D. of one experiment performed in triplicate. The experiment was repeated with similar results. Statistical analysis was performed using the Student's *t* test (two-tailed). Values of $p < 0.05$ (★) were considered significantly different.

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signaling after 24 h pre-exposure to either CPCCOEt or (*S*)-4C3HPG. Calculations of glutamate $EC_{50}$ values for the IP assays were derived from the added glutamate concentrations (Table 1). In a typical IP experiment, endogenous glutamate concentrations in the supernatant rise up to about 0.7 $\mu$M within the 30-min glutamate treatment time (Table 4). This concentration is 30 times lower than the calculated $EC_{50}$ values. The potency of CPCCOEt and (*S*)-4C3HPG to block glutamate-induced $Ca^{2+}$ mobilization was significantly decreased when cells were first pretreated with these agents (Fig. 4, Table 2). These results indicate that the antagonist action could change the receptor affinity for glutamate and antagonists or alter the efficacy of the interactions between signaling molecules in the cascade. It has been suggested that an increase in receptor agonist potency correlates with an increase in receptor number (Martin et al., 1999).

**Supersensitivity: Remember the Dopamine Receptor.** For the dopamine receptor a similar enhancement of the agonist response was observed after long-term antagonist pretreatment. This so-called dopamine receptor supersensitization was accompanied by an increase in agonist potency (Missale et al., 1989) and an apparent decrease in antagonist potency (Burt et al., 1977). The mechanism behind this phenomenon was never elucidated. Because the changes in dopamine receptor signaling have tentatively been linked to the development of severe side effects such as tardive dyskinesia (Crane, 1973; Baldessarini and Tarsy, 1980), the differences in agonist and antagonist potency seen after mGlu1 receptor antagonist treatment in vitro call for attention. If similar supersensitization occurred in vivo, prolonged antagonist treatment could be worrisome in clinical settings. The decrease in antagonist potencies over time might lead to drug tolerance and may imply the need for a dose increase after prolonged blockade of the mGlu1 receptor. The increased agonist potency may lead to exaggerated, abnormal agonist-mediated responses upon withdrawal of the antagonist.

**Antagonists Do More Than Inhibit Agonist-Induced Receptor Desensitization.** The kinetic profile of the antagonist-evoked mGlu1 receptor supersensitivity illustrated that 30-min pretreatment with either CPCCOEt or (*S*)-4C3HPG is sufficient to maximally enhance the $Ca^{2+}$ mobilization response to glutamate (Fig. 5A). Another kinetic profile was found when measuring [$^3$H]IP production (Fig. 5B). Instead of the maximum signal being reached almost immediately, glutamate-induced [$^3$H]IP production increased slowly and reached its maximum after 24-h pre-exposure to CPCCOEt. When using the 5-min glutamate treatment instead of the 30-min treatment, we did observe a significant enhancement in responsiveness after only 30-min CPCCOEt pretreatment (Fig. 6), suggesting that the 30-min glutamate treatment leads to a considerable loss of active receptors through desensitization. The discrepancy between kinetic profiles of the antagonist-mediated enhancement of changes in free [$Ca^{2+}$] in the cell and total IP levels is not surprising. It is well known that $IP_3$ molecules derived after agonist stimulation trigger a cascade of $Ca^{2+}$ signaling, leading to a measurable change in intracellular free [$Ca^{2+}$] being the net result of $Ca^{2+}$ released from $IP_3$ and ryanodine sensitive stores, $Ca^{2+}$ being buffered via $Ca^{2+}$ binding proteins and mitochondria, $Ca^{2+}$ being pumped out of the cell, and influx of $Ca^{2+}$ from the extracellular medium. Because dantrolene did not affect the glutamate-induced $Ca^{2+}$ response (data not shown), the contribution of $Ca^{2+}$ mobilized from the ryanodine sensitive pool is negligible in this cell line. Agonists have also been shown to modulate the mobilization of intracellularly stored $Ca^{2+}$ independent of PLC activation via activation of a G protein (Xu et al., 1996) or sphingosine kinase (Meyer zu Heringdorf, 1998). This theory may explain why the level of free $Ca^{2+}$ has reached a maximal steady-state level. Martin et al. (1999) described a similar nonlinear relationship between agonist-induced $IP_3$ accumulation and $Ca^{2+}$ elevation in SH-SY5Y cells. They suggested that only a limited portion of the total measured $IP_3$ has access to the $IP_3$ receptor and that this saturating amount of $IP_3$ results in maximal $Ca^{2+}$ mobilization.

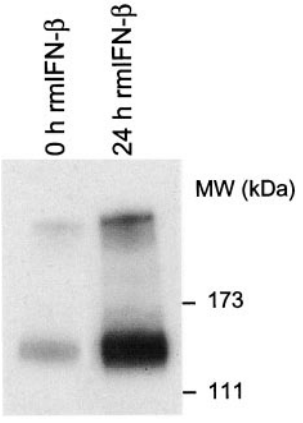


**Fig. 9.** Expression of the hmGlu1a receptor assessed by Western blot analysis. Cells were induced with rmIFN-$\beta$ for 24 h or not. Shown is an autoradiogram from a representative Western blot. Similar findings were found in two additional experiments.

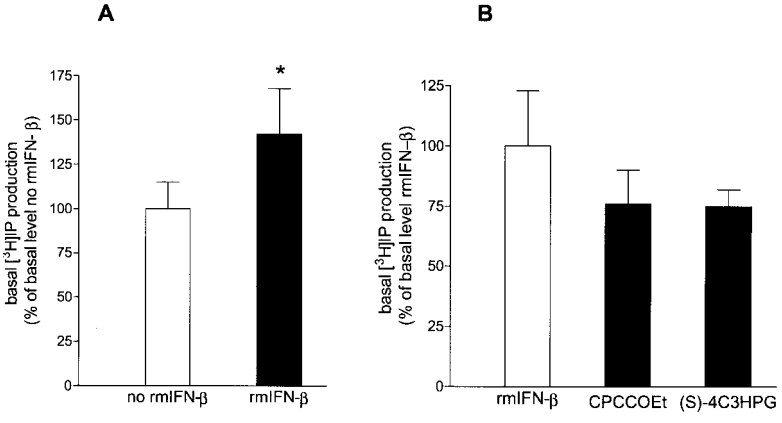


**Fig. 10.** Constitutive activity of the hmGlu1a receptor in L929sA cells. A, basal IP accumulation in noninduced and rmIFN-$\beta$–induced L929sA cells. Values are expressed as percentage of the signal seen in noninduced cells. The basal level of [$^3$H]IP accumulation in noninduced cells was 12,290 ± 1,882 d.p.m. and 14,807 ± 999 d.p.m. in cells induced with 1,000 U/ml rmIFN-$\beta$ for 24 h. B, effect of preincubation for 30 min with either CPCCOEt (100 $\mu$M) or (*S*)-4C3HPG (300 $\mu$M) on basal IP level in rmIFN-$\beta$–induced L929sA cells. Values are expressed as percentage of the signal seen in induced cells. Values are mean ± S.D. of three experiments. Values of $p < 0.05$ (★) were considered significantly different.

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Taken together, although prolonged preincubation with antagonist can lead to a continuos increase in the IP response, this does not necessarily have to be reflected in the overall $Ca^{2+}$ response.

In any case, the time course experiments revealed that 30-min antagonist pretreatment is sufficient to increase mGlu1a receptor signaling. The rapid nature of this event would suggest that fast molecular mechanisms are involved in the enhancement of mGlu1 receptor function. One such mechanism may be receptor resensitization, possibly dephosphorylation, and/or recycling kinetics. The presence of relatively high levels of extracellular glutamate favors a resensitization hypothesis because glutamate in the culture medium is likely to activate cellular effector pathways responsible for receptor desensitization. For the hmGlu1a receptor, it has indeed been reported that agonist-induced desensitization parallels agonist-mediated stimulation of the receptor (Desai et al., 1996). This is in line with our findings because glutamate-induced IP and $Ca^{2+}$ signaling start to decline (Fig. 8, A and B) as soon as glutamate concentrations reach ~2 $\mu$M, which can be enough to stimulate the receptor (Fig. 7). Treatment with mGlu1a receptor antagonists could permit receptor resensitization by blocking agonist-induced desensitization. If this were the case, removal of glutamate from the growing medium should provide the same resensitization. Therefore, we compared the effect of eliminating endogenous glutamate in the medium with GPT with the effect of mGlu1 receptor antagonist pretreatment. Our findings indicate that a medium switch with or without GPT results in a similar small enhancement of mGlu1 receptor signaling (Fig. 8, A and B). At early time-points, the GPT-induced enhancement of mGlu1 receptor IP signaling as well as the medium shift per se seem to resemble that of antagonist pretreatment (Figs. 5 and 8). Because the effect of CPCCOEt and (*S*)-4C3HPG on glutamate-induced $Ca^{2+}$ mobilization is about three times bigger than that of GPT (Fig. 5A), the antagonists seem to do more than preventing glutamate to activate and desensitize the receptor. One could speculate that the measured glutamate concentrations are not reflective of the actual glutamate concentrations in the vicinity of the cells or that another endogenous compound released by the cells can activate the receptor, an effect that could be inhibited by CPCCOEt and (*S*)-4C3HPG, but not by GPT. However, an additional observation provides further indication that antagonist exposure does more than reducing agonist-induced desensitization. Although receptor desensitization reaches 50 and 25% for IP experiments and $Ca^{2+}$ measurements, respectively (Fig. 8), glutamate-induced signaling was increased with 100% when the receptor was preexposed to antagonist (Figs. 1, 3, and 5).

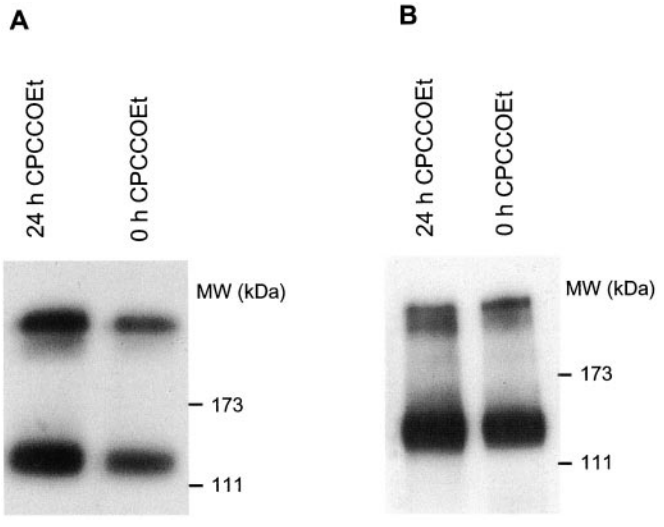


**Fig. 11.** Effect of 24 h CPCCOEt (100 $\mu$M) pretreatment of hmGlu1a receptor expressing L929sA cells on cell surface (A) versus total (B) mGlu1a receptor expression in a crude membrane preparation. Representative immunoblots are shown for mGlu1a receptor immunoreactivity after biotinylation of cell surface proteins (A) and crude membrane mGlu1a receptor immunoreactivity (B). Two additional experiments showed similar results.

Several reports showed that 24-h pretreatment with inverse agonists can lead to receptor up-regulation and subsequent enhanced responsiveness of wild-type constitutively active $\beta_2$ and $H_2$ receptors or mutationally induced constitutively active $\beta_2$ and $\alpha_{1b}$ receptors (MacEwan and Milligan, 1996; Leurs et al., 1998; Stevens et al., 2000). It was hypothesized that constitutively active receptors stimulate mechanisms responsible for desensitization and that treatment with an inverse agonist, by reducing this constitutive activity, allows receptor resensitization. Receptor up-regulation did not occur when neutral antagonists were used (MacEwan and Milligan, 1996). Therefore, we examined inverse agonist properties of the compounds under study. In L929sA cells, the hmGlu1a receptor seems to be constitutively active upon rmIFN-$\beta$-induction (Fig. 10A). This was in line with findings in LLC-PK1 and human embryonic kidney 293 cells (Prézeau et al., 1996). In rmIFN-$\beta$–induced L929sA cells, both CPCCOEt and (*S*)-4C3HPG lowered basal IP accumulation by about 25% (Fig. 10B). However, because the effect of both antagonists was not statistically significant, we cannot conclude that these compounds classify as inverse agonists or that they influence receptor activity by blocking agonist-independent receptor desensitization. Inverse agonist activities of both CPCCOEt and (*S*)-4C3HPG have been studied by others. No inverse agonist activity of CPCCOEt could be detected on native mGlu1 receptors expressed in BHK cells (Litschig et al., 1999). However, Carrol et al. (2001) recently found that this compound is able to significantly inhibit 10 to 15% of the agonist-independent activity of mGlu1 receptors boosted by coexpression with the $G_{\alpha q}$ subunit. No inverse agonist activity of (*S*)-4C3HPG has yet been detected (Prézeau et al., 1996; Carrol et al., 2001).

**Antagonist Pretreatment Increases mGlu1a Receptor Cell Surface Receptor Expression.** Treatment of L929sA cells that express the hmGlu1a receptor with CPCCOEt for 24 h does not change total mGlu1a receptor expression, as can be seen from the Western blot of a crude membrane preparation (Fig. 11B). This is in line with the fast kinetics of antagonist-mediated enhancement of mGlu1 receptor signaling, which already suggested that de novo mGlu1 receptor synthesis is not involved. Using biotinylation experiments, we demonstrate that 24-h antagonist exposure increases the amount of cell surface mGlu1a receptors (Fig. 11A). Cell surface mGlu1a receptors are enhanced upon antagonist treatment, which suggests that the antagonist may be stabilizing the receptor at or recruiting the mGlu1a receptor to the membrane. Currently, the group I mGlu receptor interacting Homer proteins are subject of intense investigation and it is becoming clear that these proteins could provide new insights into targeting and stabilization of mGlu1 receptors. Both Homer1a and Homer1c have been shown to increase mGlu1a cell surface expression in human embryonic kidney 293 cells (Ciruela et al., 1999b; 2000).

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This increase in cell surface mGlu1a receptor expression was accompanied by an increase in quisqualic acid-induced IP production. Interestingly, Homer1, -2, and -3 proteins are endogenously expressed in several mammalian cell lines (Soloviev et al., 1999). It will be interesting to know whether these proteins are expressed in L929sA cells and how antagonist blockade of the receptor affects Homer protein expression and localization.

Taken together, our data show that pretreatment of L929sA cells that express the hmGlu1a receptor with mGlu1 receptor antagonists CPCCOEt and (*S*)-4C3HPG enhances glutamate-induced [$^3$H]IP production and $Ca^{2+}$ mobilization. Because both the glutamate potency to activate PLC and the CPCCOEt and (*S*)-4C3HPG antagonist potency seem to be altered in antagonist-treated cells, we provide first evidence that the hmGlu1a receptor can supersensitize after antagonist treatment in vitro. Our results suggest that mGlu1a receptor supersensitivity is not merely the result of a blockade of agonist-mediated receptor desensitization. Although we cannot conclude that these antagonists also block agonist-independent receptor activity, it is clear that antagonist pretreatment enhances the level of mGlu1a receptors at the plasma membrane. Further studies will be necessary to examine whether mGlu1 receptor supersensitivity is accompanied by altered receptor internalization or membrane targeting, changes in the effector system coupled to the receptor (e.g., G protein, or PLC) or a facilitation of interactions in the signaling cascade.

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