Positive allosteric modulators of metabotropic glutamate 1 receptor: Characterization, mechanism of action, and binding site

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We have identified two chemical series of compounds acting as selective positive allosteric modulators (enhancers) of native and recombinant metabotropic glutamate 1 (mGlu1) receptors. These compounds did not directly activate mGlu1 receptors but markedly potentiated agonist-stimulated responses, increasing potency and maximum efficacy. Binding of these compounds increased the affinity of a radiolabeled glutamate-site agonist at its extracellular N-terminal binding site. Chimeric and mutated receptors were used to localize amino acids in the receptor transmembrane region critical for these enhancing properties. Finally, the compounds potentiated synaptically evoked mGlu1 receptor responses in rat brain slices. The discovery of selective positive allosteric modulators of mGlu1 receptors opens up the possibility to develop a similar class of compounds for other family 3 G protein-coupled receptors.

G protein-coupled receptors belong to a superfamily of integral membrane heptahelical proteins that can be activated by a large number of extracellular signals such as light, hormones, and neurotransmitters (1). Upon agonist binding, these receptors induce the activation of G proteins, which in turn transduce and amplify the signal. Based on sequence homology, these receptors have been divided into five families (1). The G protein-coupled receptor family 3 comprises receptors for extracellular calcium, pheromones, γ-aminobutyric acid (GABA, $GABA_B$ receptor) and glutamate [metabotropic glutamate (mGlu); refs. 1 and 2)]. These receptors possess a large N-terminal extracellular domain that has been demonstrated by site-directed mutagenesis, and more recently, in the case of the mGlu1 receptor by x-ray crystallography, to contain the agonist binding site (3–5). The mGlu receptor family comprises eight members to date and has been divided into three groups according to sequence homology, signal transduction mechanism, and pharmacology (2, 6). Group I contains the mGlu1 and mGlu5 receptors, which both have several splice variants. A role for mGlu1 and 5 receptor activation has been implicated in physiological processes including pain perception, learning, and memory as well as in certain psychiatric and neurological disorders (2, 7, 8). These receptors are coupled to phosphoinositide hydrolysis and can also modulate the activity of Ca^{2+} or K^+ channels, probably via the $\beta\gamma$ subunits of the heterotrimeric G protein (9). We have identified two chemical classes of small molecules that behave as selective positive allosteric modulators (enhancers) of the mGlu1 receptor. The properties of several of these compounds have been characterized extensively, and here we report their effects on recombinant and native systems and the identification of their putative binding site.

Experimental Procedures

Construction of Chimeras and Point Mutants. cDNAs encoding the rat (r)mGlu1a and rmGlu5a receptors in pBluescript II were obtained from S. Nakanishi (Kyoto, Japan). Human (hm)Glu1a receptor cDNA was amplified from a human fetal brain cDNA library in pCMV.SPORT2 (Life Technologies, Grand Island, NY) by using primers derived from the hmGlu1a sequence (AC:U31216). All point mutants were constructed by using the QuickChange site-directed mutagenesis kit (Stratagene). The rmGlu1a/5a chimeras were generated by using the PCR overlap extension method (10) or by introducing unique restriction sites that do not change amino acids at the corresponding positions in rmGlu1a and -5a and then exchanging the corresponding fragments between mGlu1a and -5a. All the PCRs were performed by using *Pfu* Turbo DNA polymerase (Stratagene). The sequence of each chimera and point mutation was confirmed by using automated cycle sequencing (Applied Biosystems).

Electrophysiology. Whole-cell voltage-clamp recordings of freshly dissociated CA3 neurons were obtained as described previously (11). Chinese hamster ovary cells stably expressing concatenated human inwardly rectifying K^+ channel (Kir)3.1 and Kir3.2c G protein-gated Kir (GIRK) channel subunits were cotransfected with a 1:1 (wt/wt) mixture of mGlu/enhanced green fluorescent protein plasmids by using Lipofectamine 2000 (Life Technologies). For electrophysiological experiments, the cells were superfused with a standard salt solution that contained 115 mM NaCl, 30 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, and 10 mM D-glucose, pH adjusted to 7.4 with NaOH and osmolarity adjusted to 340 mosmol/liter with sucrose. Pipettes were pulled from borosilicate glass with resistances ranging from 2 to 3 MΩ and filled with a solution containing 130 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 5 mM $K_4$1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate, 3 mM Na_2ATP, and 0.3 mM Na_2GTP adjusted to pH 7.2 with KOH and osmolarity adjusted to 310 mosmol/liter with sucrose. Whole-cell currents were amplified with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), filtered at 100 Hz, and digitized at 500 Hz with a Digidata 1200A acquisition board (Axon Instruments) for subsequent storage on a Dell Optiplex PC. The recordings were made under conditions in which K^+ currents would be inward ($[K^+]_i = 150$ mM, $[K^+]_o = 30$ mM, and $V_{hold} = -70$ mV). The series resistance was 5–10 MΩ. Data acquisition and analysis were performed with the PCLAMP 8.0 software package (Axon Instruments).

Stock solutions of glutamate (Fluka) and (*S*)-3,5-dihydroxy-

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Abbreviations: GABA, γ-aminobutyric acid; mGlu, metabotropic glutamate; r, rat; hm, human; Kir, inwardly rectifying K^+ channel; GIRK, G protein-gated Kir; S-DHPG, (*S*)-3,5-dihydroxyphenylglycine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ro 67-7476, (*S*)-2-(4-fluoro-phenyl)-1-(toluene-4-sulfonyl)-pyrrolidine; Ro 01-6128, diphenylacetyl-carbamic acid ethyl ester; Ro 67-4853, (9H-xanthene-9-carbonyl)-carbamic acid butyl ester; TM, transmembrane; VGCC, voltage-gated Ca^{2+} channel current; MCPG, (*S*)-α-methyl-4-carboxyphenylglycine; EPSC, excitatory postsynaptic current.

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phenylglycine (S-DHPG, Tocris Neuramin, Bristol, U.K.) were prepared in H_2O, (*S*)-2-(4-fluoro-phenyl)-1-(toluene-4-sulfonyl)-pyrrolidine (Ro 67-7476), (9H-xanthene-9-carbonyl)-carbamic acid butyl ester (RO 67-4853), and diphenylacetyl-carbamic acid ethyl ester (Ro 01-6128) in DMSO. Stock solutions were diluted in the external solutions to their final concentration before use. The maximum concentration of DMSO was 0.1%. GTP, ATP, and creatine phosphate were obtained from Roche Molecular Biochemicals; all other chemicals were obtained from Sigma.

[^{3}H]Quisqualate Binding. The effect of Ro 67-7476 and Ro 01-6128 on the binding of 10 nM [^{3}H]quisqualate to recombinant rmGlu1a and -5a was assayed as described (12). Saturation isotherms were performed by incubating 50 μg of mGlu1a receptor membrane protein with various concentrations of [^{3}H]quisqualate (1–1,000 nM final concentration, TRK 1058, Amersham Pharmacia) in the absence or presence of 0.1, 1, and 10 μM of Ro 67-7476 or Ro 01-6128. After a 1-h incubation at room temperature the membranes were filtered onto glass fiber filters (Whatman GF/C) and washed three times. Dissociation experiments were performed by incubating the membranes with 10 nM [^{3}H]quisqualate for 2 h at room temperature. Cold quisqualate (100 μM) was then added alone or in combination with 0.3 or 3 μM Ro 67-7476 or Ro 01-6128, and aliquots of membrane suspension were filtered as described above at different time intervals. In all these experiments, nonspecific binding was defined in the presence of 1 mM glutamate. Saturation isotherms and dissociation kinetics were analyzed by using one or two site models, and the results were compared by using an F test (GraphPad, San Diego).

Calcium Imaging. Transiently transfected HEK293 were incubated with 20 μM fura-2AM (Molecular Probes) for 40 min at room temperature with a 20-min postincubation in Hanks' balanced salt solution. Cells were stimulated at room temperature in artificial cerebrospinal fluid with drug as indicated. The stimuli were separated by 10-min washes. Imaging measurements were made on an inverted microscope with a long distance $\times$40 objective (Axiovert 405 M, Zeiss). A cooled charge-coupled device camera (CH-250, Photometrics, Tucson, AZ) was used to acquire image pairs at 340- and 380-nm excitation wavelengths (with dark correction) to computer. Exposure times were 400 msec. The intrinsic fluorescence in cells not dye-loaded was less than 5% and did not contribute a significant error to the measurements. Fluorescence ratio values were calculated as described (13).

Cerebellar Wedge Experiments. Sagittal slices (400 μM) were cut from the cerebellar vermis of 4–7-week-old rats by using a vibratome. The tissue was submerged at all times in an ice-cold simple salt solution containing 124 mM NaCl, 2.5 mM KCl, 2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, and 4 mM sucrose gassed with 95% O_2/5% CO_2 (pH 7.4). Cerebellar lobes were dissected from the slices and mounted in a two-compartment perspex perfusion chamber with the Purkinje cell bodies and dendrites continuously perfused with salt solution containing tetrodotoxin (300 nM) separated from the white matter tracts containing Purkinje cell axons. Population depolarizations were evoked by 1-min duration applications of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 6 μM) or S-DHPG, recorded by using Ag/AgCl/agar electrodes connected to a D.C. amplifier, and acquired by using MACLAB8 software. AMPA applications were made at the beginning and end of the experiments to control for the stability of the preparation. All experiments were performed at room temperature.

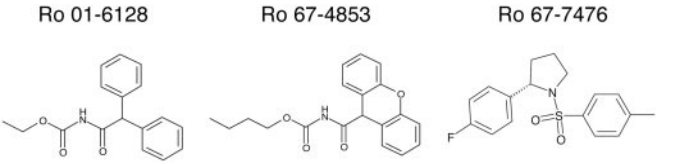

Fig. 1. Chemical structure of mGlu1 receptor enhancers.

Purkinje Cell Recordings. Transverse cerebellar slices from 15–20-day-old rats were cut in a simple salt solution (see above) by using a vibratome. Slices were maintained at 35°C for 1 h and then at room temperature. Single slices were placed in a recording chamber perfused at room temperature. Patch pipettes were pulled from thin-walled borosilicate glass (GC150TF, Clark Electromedical Instruments, Pangbourne, U.K.) by using a DMZ universal electrode puller (Zeitz Instrumente, Augsburg, Germany). Pipettes had resistances of $\approx$2–4 MΩ when filled with patch-pipette solution containing 145 mM K^+-gluconate, 2 mM $MgCl_2$, 0.75 mM EGTA, 0.1 mM $CaCl_2$, 10 mM Hepes, 2 mM Mg-ATP, and 0.3 mM Na_3GTP, pH adjusted to 7.3 with CsOH, 290 mosmol. Whole-cell recordings were made from Purkinje cell soma visualized by using a $\times$40 water immersion objective and IR-DIC optics (Olympus BX50WI, Schwerzenbach, Switzerland) using an Axopatch 1D amplifier (Axon Instruments). Access resistances were between 5 and 20 MΩ and were monitored continuously. Parallel fibers were stimulated in the cerebellar molecular layer (0.05 Hz) in bursts of 1–15 pulses (100 Hz). Synaptic events were recorded onto digital audio tape (DTR-1204, BioLogic, Claiz, France). Data acquisition and analysis was carried out by using PCLAMP7 (Axon Instruments).

Results

Effect on Recombinant rmGlu1a and hmGlu1a Receptors. Two series of positive allosteric modulators of recombinant rmGlu1a were identified by using high throughput fluorometric imaging techniques: 2-phenyl-1-benzenesulfonyl-pyrrolidine derivatives including Ro 67-7476, diphenylacetyl- and (9H-xanthene-9-carbonyl)-carbamic acid esters including Ro 01-6128 and (9H-xanthene-9-carbonyl)-carbamic acid butyl ester (Ro 67-4853; refs. 14 and 15; see Fig. 1). In HEK293 cells transiently transfected with rmGlu1a receptors (1 pmol/mg of protein), glutamate elicited a concentration-dependent increase in intracellular free calcium as assayed by single-cell fura-2 imaging (Fig. 2*A*, EC_{50} = 6.8 μM). Application of Ro 67-7476 alone did not elicit a calcium response; however, coapplication of Ro 67-7476 with 1 μM glutamate, a concentration that elicited little or no

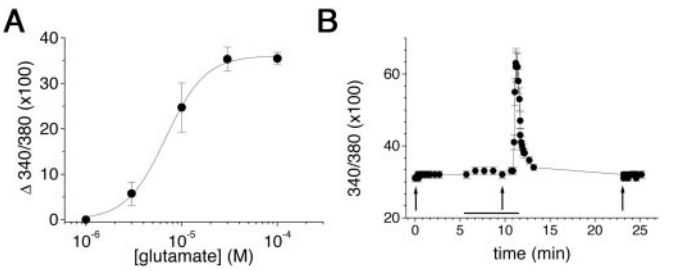

Fig. 2. Ro 67-7476 enhances glutamate-evoked increases in $[Ca^{2+}]_{int}$. (*A*) Glutamate evoked a concentration-dependent increase in $[Ca^{2+}]_{int}$ in HEK293 cells transiently transfected with rmGlu1a assayed by single-cell fura-2 imaging (data are means $\pm$ SEM of three separate experiments with 15–20 cells per experiment). (*B*) Mean data from a single representative experiment (n = 15 cells). Applications of glutamate (1 μM, 30 sec; arrows) alone elicited no response; however, application in the presence of Ro 67-7476 (bar, 1 μM), which elicited no response when applied alone, resulted in a large increase in $[Ca^{2+}]_{int}$.

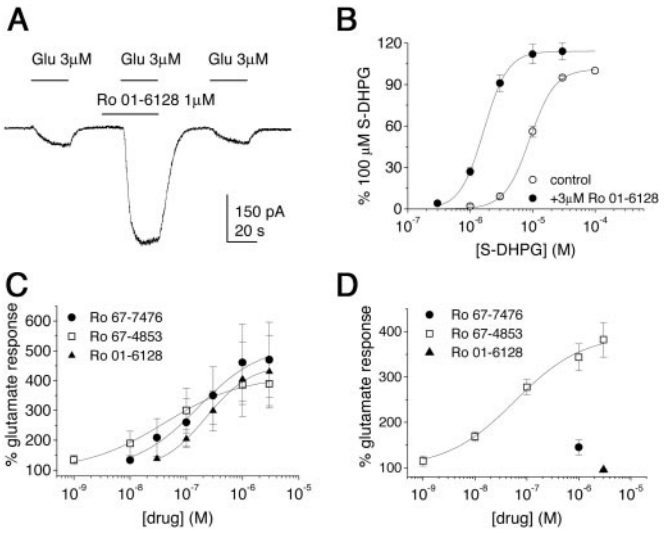

Fig. 3. Effect of enhancers on rmGlu1a and hmGlu1a receptors. (*A*) Glutamate activated an inward current in a Chinese hamster ovary cell stably expressing GIRKs and transiently transfected with the rmGlu1a receptor cDNA. The cell was held at −70 mV, and the recording was made under conditions in which K^+ currents would be inward. Ro 01-6128 alone elicited no response; however, glutamate application in the presence of Ro 01-6128 resulted in a greatly enhanced inward current. The effect of Ro 01-6128 was reversible. (*B*) Concentration-response curves for S-DHPG recorded from cells expressing rmGlu1a receptors in the absence and presence of Ro 01-6128. S-DHPG was applied for 20 sec locally to the cell with a rapid application system by using increasing concentrations. The interval between two successive applications was 90 sec. Ro 01-6128 was then added to the bath perfusion, and a second S-DHPG concentration-response curve was generated on the same cell in the presence of Ro 01-6128. The current amplitudes were normalized to the control responses and fitted individually for each cell. The pEC_{50} values for S-DHPG in the absence and presence of Ro 01-6128 were 5.05 ± 0.03 and 5.78 ± 0.05, respectively (n = 4). The effect of enhancers on glutamate-induced currents in cells expressing rmGlu1a (*C*) and hmGlu1a (*D*) receptors is shown. Maximum current amplitudes obtained at different concentrations of Ro 01-6128, Ro 67-4853, and Ro 67-7476 are plotted as a function of concentration. The values are normalized to the control responses obtained with glutamate alone (3 μM, 100%). Each point represents the mean ± SEM of five cells.

response when applied alone, resulted in a large glutamate response that recovered on wash-out of Ro 67-7476 (Fig. 2*B*). Similar enhancing effects were observed for Ro 67-4853 and Ro 01-6128 (data not shown). In cells expressing very high levels of rmGlu1a receptors (30 pmol/mg of protein, glutamate EC_{50} = 1 μM), these compounds produced an increase in $[Ca^{2+}]_{int}$ in the absence of exogenous glutamate (data not shown).

To evaluate the pharmacology of these compounds further, we used an electrophysiological assay based on the activation of GIRKs by group I mGlu receptors (16). A Chinese hamster ovary cell line stably expressing concatenated human Kir3.1 and Kir3.2c GIRK subunits was transiently transfected with rmGlu1a or hmGlu1a receptor cDNA. Rapid application of glutamate (3 μM) induced an inward current in cells expressing the rmGlu1 receptor (Fig. 3*A*). Application of 1 μM Ro 01-6128 alone had no effect; however, coapplication with 3 μM glutamate resulted in a marked potentiation of the glutamate response that recovered on wash-out of Ro 01-6128 (Fig. 3*A*). In addition, concentration-response curves by using the selective group I mGlu receptor agonist, S-DHPG, in the absence and presence of Ro 01-6128 (3 μM) revealed an approximate 5-fold decrease in the agonist EC_{50} value and 1.2-fold increase in the maximum response amplitude in the presence of the enhancer (Fig. 3*B*). We generated concentration-response curves for the potentiation of 3 μM glutamate-induced currents by the compounds at the rmGlu1a receptor (Fig. 3*C*). The pEC_{50} values were 6.68 ± 0.12, 7.16 ± 0.20, and 6.76 ± 0.08 for Ro 01-6128, Ro 67-4853, and Ro 67-7476, respectively (n = 5–8). Interestingly, in hmGlu1a receptor-expressing cells, Ro 67-7476 and Ro 01-6128 had little or no effect on the glutamate-induced current, whereas Ro 67-4853 produced a pronounced enhancement (Fig. 3*D*).

Selectivity. By using various functional models including GTPγ[^{35}S] binding, $[Ca^{2+}]_{int}$ imaging, and activation of GIRKs it was found that these compounds are devoid of any enhancing effect at recombinant rmGlu2, rmGlu4, and rmGlu8 and human $GABA_B$ receptors. Importantly, Ro 67-7476 (1 μM) and Ro 01-6128 (10 μM) did not enhance the glutamate-induced GIRK current in rmGlu5a receptor-expressing cells. In contrast, a small potentiating effect was observed with Ro 67-4853 (10 μM) at this receptor (data not shown). In addition, we did not observe any modulatory effects at the M1 muscarinic receptor, which is expressed endogenously in HEK293 cells or at recombinant D2 dopamine receptors. At a high concentration, Ro 67-7476 (10 μM), but not Ro 01-6128 or Ro 67-4853, directly blocked the GIRK channel (to 67 ± 2% of control, n = 4). Finally, Ro 67-7476 and Ro 67-4853 (10 μM) exhibited no activity in radioligand binding assays at adenosine A1 or A2; adrenergic α1, α2, β1, or β2; $GABA_A$; glycine; histamine H1; muscarinic M1, M2, or M3; nicotinic; opiate; purinergic P2x and 5-serotonin receptors and adenosine; norepinephrine; or GABA and 5-serotonin uptake sites (data not shown).

Chimeras and Point Mutants. To localize structural determinants that mediate the enhancing effect of Ro 67-7476 in rmGlu1, we constructed chimeric rmGlu1a/rmGlu5a receptors (Fig. 4*A*). We examined the enhancement of glutamate-induced GIRK currents at these chimeric receptors. Ro 67-7476 enhanced the glutamate-induced current in all chimeric receptors containing the transmembrane (TM) region of rmGlu1a (R1, R1-R5N, R1-R5C, and R5-R1TM) but not in those containing the TM region of rmGlu5a (R5, R5-R1N, R5-R1C, and R1-R5TM; Fig. 4*C*). Therefore, the TM region of rmGlu1a is required for the enhancing effect of Ro 67-7476. Sequence alignment of the TM regions of rmGlu1a and rmGlu5a revealed that many amino acids differ between the two receptors. Interestingly, only one amino acid in the TMV region (position 757) differs between rmGlu1a V757 and hmGlu1a L757, and this leucine is conserved in rmGlu5a (L743; Fig. 4*B*) and in all other mGlu receptors. Because Ro 67-7476 (1 μM) had no enhancing activity at both hmGlu1a (H1) and rmGlu5a (R5), we reasoned that this valine could be critical for the enhancing effect. Therefore, we constructed valine to leucine (rmGlu1a, R1-V757L) or leucine to valine (hmGlu1a, H1-L757V, and rmGlu5a, R5-L743V) mutants at this residue. In the rmGlu1a R1-V757L mutant, the enhancing effect of Ro 67-7476 was abolished, whereas a robust enhancement was evident in the hmGlu1a mutant H1-L757V. Thus, a valine at position 757 of rmGlu1a is critical for the enhancing effect of Ro 67-7476. Only a very weak effect was observed with Ro 67-7476 in the rmGlu5a R5-L743V mutant (Fig. 4*D*), suggesting that other nonconserved residues are likely to be critical for the enhancing effect in rmGlu1a. To identify these critical amino acids we performed additional mutagenesis of nonconserved TM residues. Double mutation of two amino acids critical for the effect of the mGlu1 receptor antagonist CPCCOet (17), R1-T815M,A818S did not alter the enhancing effect of Ro 67-7476 (Fig. 4*D*). However, a triple mutant of rmGlu5a carrying mutated residues at the mGlu5 antagonist 2-methyl-6-(phenylethynyl)pyridine binding site (18), P654S,S657C, together with the leucine to valine, L743V, conferred a strong enhancing effect of Ro 67-7476, indistinguishable from that on the wild-type rmGlu1a receptor. Mutation of the corresponding residues in the rmGlu1 receptor, R1-S668P,C671S, resulted in a

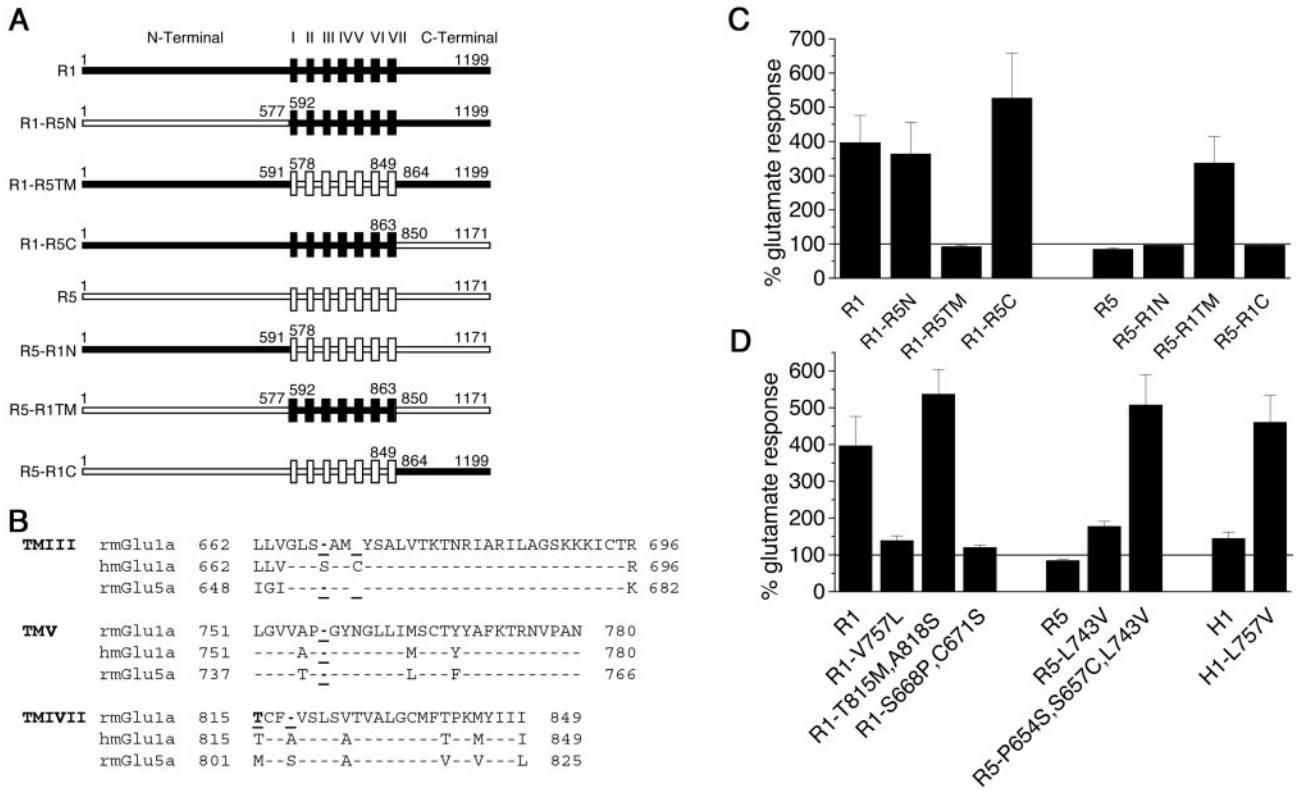

Fig. 4. A series of chimeric receptor constructs showing the fusion sites between rmGlu1a (R1, black) and rmGlu5a receptors (R5, white). (*A*) The numbering indicates amino acid fragments of wild-type rmGlu1a and rmGlu5a receptors. (*B*) Sequence alignment of the TMIII, TMV, and TMVII domains of the rmGlu1a and hmGlu1a receptors with rmGlu5a receptor, showing the nonconserved valine at position 757. The residues that were mutated in the current work are underlined. – indicates identical residues among rmGlu1a, hmGlu1a, and rmGlu5a receptors. Enhancement of the glutamate-induced current in GIRK-Chinese hamster ovary cells expressing chimeric (*C*) and mutated (*D*) rmGlu1a (R1), hmGlu1a (H1), and rmGlu5a (R5) receptors by 1 μM Ro 67-7476. To achieve a comparable activation of the receptors, glutamate concentrations approaching the EC_{20} values were used (1 μM for rmGlu5a- and 3 μM for hmGlu1a- and rmGlu1a N-Terminal-containing receptors). Data are expressed as percentages of the maximum current amplitude induced by a control application of glutamate alone and represent the mean ± SEM of five to eight cells.

loss of the enhancing effect of Ro 67-7476 at this receptor (Fig. 4*D*). Identical results were obtained with Ro 01-6128 (data not shown).

Effect on [^{3}H]Quisqualate Binding. It has been demonstrated that the mGlu1 N-terminal extracellular domain contains the agonist (glutamate) binding site (5). The effect of Ro 67-7476 and Ro 01-6128 was tested on the binding of the high affinity agonist, [^{3}H]quisqualate, to recombinant rmGlu1a and rmGlu5a receptors. There was no significant effect of these two compounds, up to concentrations of 10 μM, on the binding of 10 nM [^{3}H]quisqualate to rmGlu5a receptor, whereas they increased the binding of this ligand to the rmGlu1a receptor (data not shown). In saturation experiments, both compounds concentration-dependently increased [^{3}H]quisqualate affinity for the mGlu1a receptor with a 2.3- and 1.5-fold decrease in the dissociation constant (K_D) values in presence of 10 μM Ro 67-7476 and Ro 01-6128, respectively (Table 1). All the saturation isotherms were better fitted by a one- rather than a two-site model, and at the highest concentration of the enhancers there was a trend toward an increase of the maximum number of binding sites (B_{max}). An assay of the effect of the compounds on quisqualate dissociation kinetics revealed a one- rather than two-phase dissociation, and at 3 μM both compounds reduced the dissociation rate constant K_{off} parameter by 1.5–1.7-fold (Table 1).

Effect on Native mGlu1 Receptors in Rat Brain Neurons. It has been shown previously that activation of group I mGlu receptors inhibits voltage-gated Ca^{2+} channel currents (VGCCs) in hippocampal CA3 neurons (19, 20). The effect of the mGlu1 receptor enhancers therefore was investigated on VGCC inhi-

Table 1. Dissociation constant, K_D, maximum number of binding sites, B_{max}, and dissociation rate constant, k_{off}, parameters obtained from [^{3}H]quisqualate saturation isotherms and dissociation kinetic experiments with rat mGlu1a receptors in the absence (control) or presence of 0.1, 0.3, 1, 3, and 10 μM Ro 67-7476 or Ro 01-6128

	K_D ± SD, nM	B_{max} protein ± SD, pmol/mg	K_{off} ± SD, min^{-1}
Control	29 ± 8	6 ± 0.3	0.14 ± 0.03
Ro 67-7476 0.1 μM	26 ± 9	6 ± 0.35	ND
Ro 67-7476 0.3 μM	ND	ND	0.16 ± 0.11
Ro 67-7476 1 μM	16 ± 2.5*	6.5 ± 0.42	ND
Ro 67-7476 3 μM	ND	ND	0.075 ± 0.04*
Ro 67-7476 10 μM	12 ± 4†	6.7 ± 0.37	ND
Ro 01-6128 0.1 μM	30 ± 15	6 ± 0.36	ND
Ro 01-6128 0.3 μM	ND	ND	0.096 ± 0.034
Ro 01-6128 1 μM	20 ± 2*	7.6 ± 0.5	ND
Ro 01-6128 3 μM	ND	ND	0.1 ± 0.014*
Ro 01-6128 10 μM	18.5 ± 5*	7.5 ± 0.5	ND

The results represent the mean ± SD of at least three individual experiments performed in triplicate; ND, not determined.
*, $P < 0.05$ vs. control, Student's *t* test.
†, $P < 0.01$ vs. control, Student's *t* test.

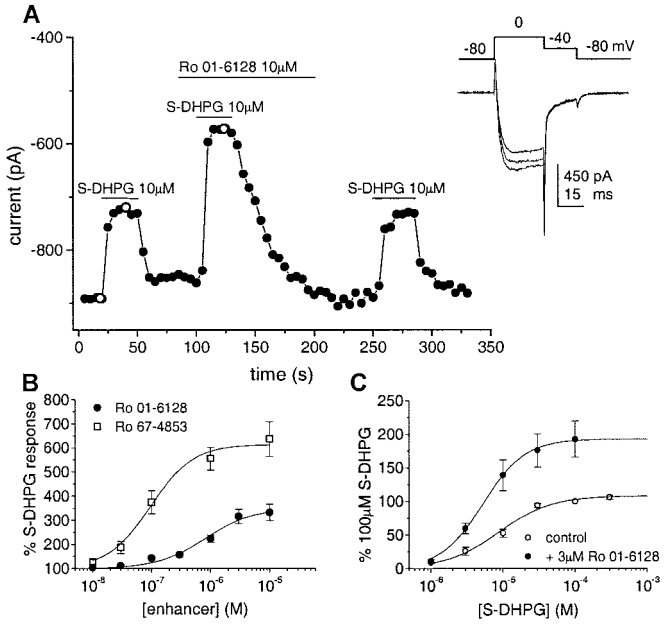

Fig. 5. The effect of enhancers in freshly dissociated CA3 neurons. (*A*) VGCCs were evoked at 5-sec intervals by 30-msec steps to 0 mV from a holding potential of −80 mV. The currents measured at the peak of the control response and their inhibition by S-DHPG in the presence and absence of Ro 01-6128 are plotted versus time. S-DHPG and Ro 01-6128 were applied for the times indicated by the bars. (*Inset*) Superimposed current traces generated in the absence and presence of S-DHPG and Ro 01-6128, represented by the open circles in the plot. (*B*) Concentration-response curves for the enhancement of the S-DPHG response by Ro 01-6128 and Ro 67-4853 in freshly dissociated CA3 neurons. VGCCs were evoked at 5-sec intervals by 30-msec steps to 0 mV from a holding potential of −80 mV. The currents measured at the peak of the control response and their inhibition by S-DHPG (5 μM) were recorded in the presence and absence of the different concentrations of the enhancers. The values for S-DHPG inhibition were normalized to the control responses and fitted individually for each cell. (*C*) Concentration-response curves for S-DHPG in freshly dissociated CA3 neurons in the absence and presence of 3 μM Ro 01-6128. S-DHPG was applied for 20 sec locally to the cell with a rapid application system by using increasing concentrations. The interval between two successive applications was 90 sec. Ro 01-6128 was then added to the bath perfusion, and a second S-DHPG concentration-response curve was generated on the same cell in the presence of Ro 01-6128. The current amplitudes were normalized to the control responses and fitted individually for each cell. The pEC_{50} values for S-DHPG in the absence and presence of Ro 01-6128 were 5.05 ± 0.07 and 5.32 ± 0.05, respectively (n = 5).

bition by S-DHPG in freshly dissociated CA3 neurons. As shown in Fig. 5*A* for a representative cell, Ro 01-6128 (10 μM) potentiated the inhibitory effect of S-DHPG (mean potentiation, 227 ± 21%, n = 12) but was devoid of any effect when applied alone. Ro 01-6128 also enhanced glutamate responses evoked in the presence of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoxaline (20 μM) and (*R*)-3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonate (20 μM) to block the ionotropic glutamate receptors (data not shown). However, inhibition of VGCCs via activation of group II mGlu receptors by LY354740 (1 μM) in Golgi cells of the rat cerebellum (21) was not affected by 3 μM Ro 01-6128. The values for the inhibition of the control current by LY354740 were 31.2 ± 4.5% and 31.5 ± 4.0% (n = 4) in the absence and presence of Ro 01-6128, respectively.

Concentration-response curves for the effect of Ro 01-6128 and Ro 67-4853 were generated in CA3 neurons by using 5 μM S-DHPG (EC_{30}, Fig. 5*B*). The maximum potentiation of the S-DHPG effect by Ro 01-6128 and Ro 67-4853 were 333 ± 34% and 637 ± 72% of control, respectively. Ro 67-4853 was more potent in enhancing the effect of S-DHPG than Ro 01-6128, with

Table 2. Characterization of mGlu receptor enhancers in CA3 neurons

	Max	pEC_{50}	n
Ro 01-6128	375 ± 40	5.97 ± 0.11	0.82 ± 0.05
Ro 67-4853	611 ± 59	7.02 ± 0.06	1.3 ± 0.09

Potentiation of the inhibition of VGCCs by S-DHPG was determined in the presence of different drug concentrations ([drug]) and expressed as a percentage of the control inhibition (5 μM S-DHPG). The data were fitted with the equation $Y = 100 + (\text{Max} - 100)/(1 + (EC_{50}/[\text{drug}])^n)$, where Max is the maximum effect, EC_{50} is the concentration eliciting a half-maximum effect, and n is the Hill slope. The data represent the mean ± SEM of 5–7 cells.

EC_{50} values of 95 nM and 1.1 μM, respectively (Table 2). At the highest concentration tested (10 μM), Ro 01-6128 slightly inhibited the VGCCs in the absence of S-DHPG (2.3 ± 0.2%, n = 5), whereas Ro 67-4853 had no such direct effect. We also generated S-DHPG concentration-response curves in the absence and presence of 3 μM Ro 01-6128 (Fig. 5*C*). The maximum S-DHPG-evoked inhibition increased by 1.8-fold, and the EC_{50} decreased by 1.8-fold in the presence of the enhancer. Thus, in both native and recombinant systems, enhancer binding increased both agonist potency and maximum efficacy.

Enhancing Properties of Ro 67-7476 on Synaptic Transmission in Rat Cerebellar Slices. mGlu1 receptors are expressed at high levels in cerebellar Purkinje cells (22), and exogenous application of mGlu1 agonists to cerebellar slice preparations elicits an (*S*)-α-methyl-4-carboxyphenylglycine (MCPG)-sensitive inward current (23), which is absent in mGlu1−/− animals (24). We used a greased-gap recording technique to elicit mGlu1-mediated inward currents with S-DHPG in a cerebellar wedge preparation. S-DHPG-induced population depolarizations were potentiated significantly in the presence of Ro 67-7476 (3 μM; Fig. 6*A*), whereas control AMPA (6 μM) responses were unaffected (data not shown). To investigate the effects of Ro 67-7476 on synaptically activated mGlu1 receptors, we studied the MCPG-sensitive Purkinje cell postsynaptic inward current activated by repetitive parallel fiber stimulation and likely to be mediated via mGlu1 activation (25, 26). Under control conditions, the am-

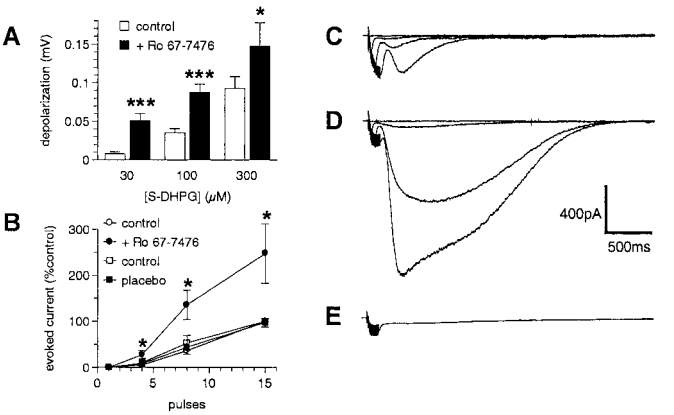

Fig. 6. Potentiation of mGlu receptor responses in cerebellar slices by Ro 67-6747. (*A*) Mean (± SEM) population depolarizations evoked by the application of S-DHPG to cerebellar wedges in the presence or absence of Ro 67-7476 (3 μM), n = 6–22 (*, $P < 0.05$, ***, $P < 0.001$, paired Student's t test). (*B*) Mean mGlu receptor EPSC amplitude increased as a function of stimulation number and was potentiated after the application of Ro 67-7476 (3 μM), n = 5, but was stable after a 20-min placebo incubation, n = 4. Representative mGlu receptor EPSC was evoked in response to 1, 4, 8, and 15 stimulation pulses: *C*, control; *D*, in the presence of Ro 67-7476 (3 μM); *E*, in response to 15 pulses in the presence of Ro 67-7476 + 1 mM MCPG.

plitude of the mGlu1 excitatory postsynaptic potential (EPSC), evoked in the presence of 10 μM 2,3-dihydroxy-6-nitro-7-sulfamoylbenzoquinoxaline, 50 μM picrotoxin, and 10 μM AP5 increased as a linear function of the number of pulses (1–15,100 Hz) within a burst stimulation (Fig. 6 *B* and *C*). The application of Ro 67-7476 (3 μM) produced no effect alone but resulted in a marked potentiation of the mGlu1 EPSC amplitude and duration after 20 min of bath perfusion (Fig. 6 *B* and *D*). The mGlu1 EPSC was otherwise stable over the same time period in the absence of compound (Fig. 6*B*). In keeping with previous reports, the mGlu1 EPSC was blocked completely by the application of 1 mM MCPG (Fig. 6*E*). AMPA-mediated EPSCs were unchanged in the presence of Ro 67-7476 (data not shown).

Discussion

Our results demonstrate for the first time the existence of selective allosteric enhancers of the mGlu1 receptor. These compounds, which belong to two different chemical classes, were identified initially by using recombinant mGlu1 receptors expressed at very high levels. Under these conditions, which are likely to lead to a marked level of constitutive receptor activation (27), the compounds could elicit a response in the absence of glutamate site ligands. Importantly, in native experimental systems and recombinant systems with a lower receptor expression level, the compounds did not produce a direct activation of the receptor but potentiated the agonist-stimulated response. In agreement, in cells expressing high levels of rmGlu1b receptors, but that exhibit very low constitutive activity (27), a pure enhancing activity was observed (data not shown). Accordingly, we speculate that these enhancers bind to and stabilize activated receptor states, which might include both agonist-bound states and a nonliganded, closed conformation (5) mediating constitutive activity (27). Such a mode of action would be consistent with the observed increases in glutamate site ligand potency and maximum efficacy. Our results with chimeric receptors clearly demonstrate the localization of the enhancer binding site within the TM domain of the mGlu1 receptor. By site-directed mutagenesis, we identified an amino acid in TMV of the receptor (V757), which appears to gate the enhancing effect of both Ro 01-6128 and Ro 67-7476. We also have demonstrated the involvement of critical amino acids in TMIII, previously identified as the 2-methyl-6-(phenylethynyl)pyridene binding site, at homologous residues in the mGlu5 receptor (18). It is interesting to note that these TMIII residues together with residues in TMVII appear to form an overlapping binding pocket in homologous regions of the mGlu1 and mGlu5 receptors for the selective antagonists CPCCOet and 2-methyl-6-(phenylethynyl)pyridine, respectively (17, 18). This region may represent a site for both positive and negative allosteric modulation. Definitive identification of the enhancer binding site awaits the development of an appropriate radioligand. However, our mutagenesis data together with the absence of activity at other Gq-coupled receptors strongly suggests that the enhancing effect results from a direct compound interaction with the mGlu1 receptor. In particular, imparting activity to the closely related rmGlu5 receptor by mutagenesis demonstrates that this effect does not result from a specific action on mGlu1 coupling or intracellular signaling cascade. Ro 67-4853, which is structurally different from Ro 67-7476, exhibited activity at all group I mGlu receptors including hmGlu1, rmGlu1, and rmGlu5, suggesting a different binding mode for this compound.

Although allosteric modulators of G protein-coupled receptors have been reported (28–32), such a robust enhancement as observed here has not been described. Positive allosteric modulation is an attractive mechanism for enhancing appropriate physiological receptor activation, and the results obtained in cerebellar slices strongly suggest that this class of compound can modulate physiological mGlu1 activity in the brain. Similar positive allosteric modulation of ligand-gated $GABA_A$ receptors has proven to be of important therapeutic utility. The discovery of selective mGlu1 enhancers opens the possibility for therapeutically relevant positive modulation of family 3 G protein-coupled receptors.

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