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Functional Coupling of Rat Group II Metabotropic Glutamate Receptors to an ω-Conotoxin GVIA-Sensitive Calcium Channel in Human Embryonic Kidney 293 Cells

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SUMMARY

Metabotropic glutamate receptors are G protein-coupled receptors that perform a variety of modulatory roles in the central and peripheral nervous systems. The development of receptor subtype-specific agonists/antagonists has lagged far behind the isolation and characterization of receptor cDNAs. Furthermore, the coupling of specific metabotropic receptors to the various neuronal-specific effector molecules, such as voltage-gated Ca^{2+} channels, has not been well studied. It was recently demonstrated that a rat group II metabotropic receptor (rmGluR2) is capable of coupling to endogenous N-type Ca^{2+} channels when heterologously expressed in adult rat sympathetic ganglia neurons. To eventually understand the molecular aspects of metabotropic receptor modulation of the N-type Ca^{2+} channel, we have transiently expressed both group II receptors in a human embryonic kidney 293 cell line (G1A1) that stably expresses the human α_{1B-1}, α_{2b}, and β_{1-3} Ca^{2+} channel subunits. rmGluR2 and rmGluR3 modulate the ω-conotoxin GVIA-sensitive Ba^{2+} currents in G1A1 cells using a voltage-dependent mechanism via an endogenous pertussis toxin-sensitive G protein. Cell-attached "macropatch" recordings demonstrate that modulation by rmGluR2 and rmGluR3 is membrane delimited. This is the first report of Ca^{2+} channel modulation mediated by rmGluR3. In addition, an extensive pharmacological comparison between rmGluR2 and rmGluR3 reveals that these group II receptors interact with agonists and antagonists in unique ways.

As the major excitatory neurotransmitter in the central nervous system, glutamate exerts its effects via two families of receptors. The *N*-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, and kainate receptor family are ligand-gated ion channels and are responsible for the fast excitatory effects of L-glutamate release at the synapse. Conversely, the mGluR family is characterized by seven-transmembrane spanning segments and modulates a number of intracellular signal transduction pathways via activation of heterotrimeric G proteins. To date, eight different mGluR subtypes (mGluR1-8) have been cloned from rat and subsequently expressed in various cell lines. These receptors can be grouped according to the signal transduction pathways to which they couple (reviewed in Ref. 2): group I mGluRs [mGluR1, including three splice variants (mGluR1a–c), and mGluR5, including two splice variants (mGluR5a-b)] stimulate inositol phosphate metabolism and mobilization of intracellular Ca^{2+}, whereas mGluR2-4 and mGluR6-8 negatively couple to adenylyl cyclase and are further subdivided into group II (mGluR2 and mGluR3) and group III (mGluR4 and mGluR6-8) receptors based on amino acid sequence similarity and agonist pharmacology (see below). Nevertheless, the pharmacology of mGluRs is only partially refined, and studies that identify specific physiological roles for a given mGluR in the central nervous system have lagged far behind the isolation of cDNA clones. With very few exceptions (3) it has been possible to assign the involvement only of a group of receptors and not of a specific receptor to physiological responses initiated by mGluR agonists (4, 5).

We expressed both of the group II rmGluRs (rmGluR2 and rmGluR3) in an HEK 293 cell line (G1A1) that stably ex-

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; rmGluR, rat metabotropic glutamate receptor; HEK, human embryonic kidney; t-ACPD, *cis*-(±)-1-amino-1,3-cyclopentane-dicarboxylic acid; L-AP4, L-2-amino-4-phosphonobutyric acid; L-SOP, L-serine-*O*-phosphate; L-CCGI, (2*S*,1′*S*,2′*S*)-2-(carboxycyclo-propyl)glycine; (*S*)-4C3HPG, (*S*)-4-carboxy-3-hydroxyphenylglycine; (+)-MCPG, (+)-α-methyl-4-carboxyphenylglycine; MCCG, α-methyl-(2*S*,1′*S*,2′*S*)-2-(carboxycyclo-propyl)glycine; M3C4HPG, (*R*,*S*)-α-methyl-3-carboxy-4-hydroxyphenylglycine; TEA, tetraethylammonium hydroxide; NEM, *N*-ethylmaleimide; PTX, pertussis toxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CMV, cytomegalovirus; NMG, *N*-Methyl-D-glucamine.

presses a neuronal-specific effector, the ω-conotoxin GVIA-sensitive Ca^{2+} channel. Because this effector is known to be modulated by mGluRs in acutely isolated central neurons (6, 7) and may play a central role in the modulation of synaptic transmission (8, 9), this heterologous system will likely yield information about mGluR pharmacology and function that is relevant for more physiological systems.

Materials and Methods

Isolation of cell lines stably expressing the ω-conotoxin GVIA-sensitive Ca^{2+} channel. Stable cell lines were obtained as described previously (10). Briefly, HEK 293 cells were cotransfected with pMCIneo (Stratagene, La Jolla, CA) and three pcDNA1 (InVitrogen, San Diego, CA) expression constructs containing cDNAs for the $\alpha_{1B\text{-}1}$, α_{2b}, and $\beta_{1\text{-}3}$ human Ca^{2+} channel subunits using a Ca^{2+} phosphate-mediated procedure (11). G418 (0.5 mg/ml; GIBCO, Grand Island, NY)-resistant colonies were isolated with cloning cylinders and screened for expression using both mRNA analysis and ^{125}I-ω-conotoxin GVIA binding (11). Clones that highly expressed the channel subunits were recloned by limiting dilution (12) and screened monthly for 6 months using binding, mRNA expression, and electrophysiological criteria. One cell line, G1A1, was used for the studies described below.

Cell culture and transfections. G1A1 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with bovine calf serum (5.5%; Hyclone Laboratories, Logan, UT), 100 units/ml penicillin G/ 100 mg/ml streptomycin (GIBCO), and 0.5 mg/ml G418 (Sigma Chemical, St. Louis, MO) with a 37° and 5% CO_2 incubator. Confluent cultures were seeded onto 35-mm dishes, supplemented with conditioned media, and allowed to grow ≥2 days before transfection with mGluR expression constructs (see below). At the time of transfection, cultures were usually ~30% confluent. Expression constructs (4 μg of DNA total/transfection) were introduced into these cultures using a Ca^{2+} phosphate-mediated procedure (11) except that cultures were grown for 12–18 hr in a 3% CO_2 atmosphere after the addition of the DNA/$CaPO_4$ precipitate. After overnight incubation at 3% CO_2, transfected cells were rinsed with Dulbecco's phosphate-buffered saline (Sigma), fed with a mixture of normal and conditioned media, and allowed to grow an additional 18–24 hr before the electrophysiological experiments.

To ensure proper clamp control, well-isolated, spherical G1A1 cells were obtained by removal from their dishes using a brief incubation with Dulbecco's phosphate-buffered saline containing 3 mM EDTA. After repeated, forceful washing with the Dulbecco's phosphate-buffered saline/EDTA solution, the cell suspension was pelleted, and the pellet was suspended in the original medium. Any remaining clumps of cells were dispersed by gentle trituration with a fire-polished Pasteur pipette. The final cell suspension was plated onto 35-mm dishes previously coated with poly-D-lysine, and the cells were allowed to settle in the incubator for ~10 min before use.

Molecular biology. For expression in mammalian cells, the cDNA for rmGluR2 was obtained from two independent sources. One rmGluR2 cDNA was generously provided by S. Nakanishi (Institute for Immunology, Kyoto University Faculty of Medicine, Kyoto, Japan). The second rmGluR2 cDNA was isolated, along with the rmGluR3 cDNA, from an oligo(dT)-primed cDNA library in the Lambda-Zap vector (Stratagene) constructed from cerebellar mRNA using methods reported elsewhere (13, 14). For transient expression in cell lines, rmGluR2 cDNA was subcloned 3′ of the CMV transcriptional promotor contained in two eukaryotic expression vectors, pRcCMV (InVitrogen) or pRK. For the pRcCMV construct, the *Hin*dIII/*Xba*I fragment of the Nakanishi clone was subcloned using a similarly digested vector. For the pRK construct, the cerebellar cDNA was subcloned using the *Eco*RI restriction sites in both the vector and cDNA. Although a larger percentage of expressing cells was seen for transfections with the pRK/rmGluR2 construct, the two rmGluR2 cDNAs in pRcCMV or pRK produce functionally identical receptors (data not shown) and will be referred to throughout the text as rmGluR2. To express rmGluR3, the 5′-untranslated region was removed from the cerebellar cDNA by ligation of a blunted (Klenow) *Bam*HI site in the rescued pBluescript (Stratagene) vector to the *Eco*RV site located in the untranslated region 28 bases upstream of the ATG start codon. To remove the 3′-untranslated region, an *Eco*RI site was introduced 73 bases downstream of the stop codon using conventional polymerase chain reaction techniques. An *Eco*RI fragment containing the entire rmGluR3 coding region was then subcloned into the pRK expression vector.

Plasmid DNAs were prepared for transfection from large-scale preparations using Qiagen-tip 500 columns according to the manufacturer's instructions (Qiagen, Studio City, CA).

Electrophysiology. All recordings were performed at ambient room temperature with standard patch-clamp techniques (15) using the Axopatch-1D amplifier (Axon Instruments, Burlingame, CA) in the voltage-clamp mode. Gigaohm seals were formed using patch pipettes made from borosilicate glass (World Precision Instruments, New Haven, CT). For whole-cell patch clamp recording, patch pipettes typically had input resistances of 0.5–2 MΩ using the NMG/TEA internal solution (see below). Whole-cell capacitance (typically 10–40 pF) and series resistance (typically <10 MΩ) were compensated manually after opening the cell. For cell-attached macropatch recordings, patch pipettes had input resistances of 0.03–0.05 MΩ using another TEA/Ba^{2+} external solution (see below). All currents were on-line leak-subtracted using a p/n protocol and low-pass filtered (three-pole Bessel filter) at 1–5 kHz with >70% compensation. Depolarizing test pulses were typically given at 0.25 Hz to prevent prolonged channel inactivation. Data were digitized up to 10 kHz with a Labmaster DMA (Axon), stored on a 486 microprocessor, and analyzed off-line using pClamp software (Axon). Unless otherwise stated, current amplitudes were measured from cursers placed immediately before and 15 msec after the initiation of a depolarizing test pulse. Numerical analysis was performed using the Quattro Pro software package (version 5.00; Borland International, Buffalo, NY). Current traces and concentration-response curves were generated using GraphPAD Prism (version 1.03; GraphPAD Software, San Diego, CA). Statistical analysis was performed using InStat (version 2.05; GraphPAD).

Cells were continuously bath perfused with an extracellular solution consisting of 150 mM NaCl, 10 mM dextrose, 10 mM HEPES, 2.5 mM KCl, 2.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.4 with NaOH, and osmolarity adjusted to 320–340 mmol/kg with sucrose. To isolate currents mediated by the Ca^{2+} channels, cells were locally perfused with 140 mM TEA, 10 mM HEPES, 15 mM dextrose, and 5 mM $BaCl_2$, pH 7.35 with methanesulfonic acid, and osmolarity adjusted to 320–330 mmol/kg with sucrose. L-Glutamate hydrochloride, t-ACPD, (+)-quisqualic acid (all from Research Biochemicals), L-AP4, L-SOP (both from Sigma), (*S*)-4C3HPG, and L-CCGI (both from Tocris Cookson, St. Louis, MO) were stored as concentrated stocks at −20°. L-CCGI was stored in small aliquots that were discarded after use to avoid freeze/thaw cycles. The antagonists, (+)-MCPG, M3C4HPG, and MCCG (all from Tocris Cookson), were suspended in the TEA/Ba^{2+} external solution at a working concentration and briefly sonicated to effect dissolution. Agonists and antagonists were typically applied for ≥10 sec and no longer than 30 sec from an array of 12 high performance liquid chromatography-grade capillary tubes (150-mm i.d.; Hewlett Packard Analytical Direct, Wilmington, DE) placed within 50–100 μm of the cell of interest.

For experiments in which the whole-cell configuration of the patch-clamp technique was used, the internal solution in the patch pipette was similar to that reported previously (1) and contained 125 μM *N*-methylglucamine, 20 mM TEA-OH, 14 mM $Tris_2$-phosphocreatine, 11 mM EGTA, 1 mM $CaCl_2$, 4 μM Mg-ATP, 0.3 mM Tris-GTP, pH 7.2 with methanesulfonic acid, and HCl (final [Cl], 10 mM), adjusted to 300–310 mmol/kg with sucrose. For experiments using the cell-attached macropatch configuration (15), recordings were

first made in the whole-cell configuration using the NMG/TEA internal to verify that a given cell expresses the mGluR of interest. The pipette was then carefully lifted off the cell, allowing the membrane to reseal, if possible, and the same cell was patched in the cell-attached configuration. The external solution in the cell-attached patch pipette consisted of 110 mM TEA, 8 mM HEPES, 12 mM dextrose, and 20 mM $BaCl_2$, with osmolarity of 300–310 mmol/kg and pH 7.35 with methanesulfonic acid. All currents recorded in the cell-attached configuration exhibited typical activation and time-dependent inactivation kinetics. In the cells for which it was tested, the cell-attached current-voltage relationship was characterized by a shape identical to that seen for whole-cell currents.

Results

Characterization of the stably expressed Ca^{2+} channel. It has been reported that transient expression of the human α_{1B-1}, α_{2b}, and β_{1-3} Ca^{2+} channel subunits in HEK 293 results in robust expression of functional Ca^{2+} channels with properties similar to those found for N-type Ca^{2+} channels in endogenous systems (16, 17). To demonstrate that these properties did not change appreciably by generation of a stable cell line, we examined the attributes of the stably expressed Ca^{2+} channels. First, from hyperpolarized holding potentials (−90 mV), depolarizing voltage steps to −10 mV caused inward Ba^{2+} currents that were generally characterized by rapid, exponential activation (τ = 4.4 ± 0.3 msec, n = 16; data not shown) and a rapidly deactivating tail current on return to the hyperpolarized holding potential. Second, in a majority of cells tested (35 of 44), prolonged depolarizing voltage steps (400 msec) to −10 mV caused the inward Ba^{2+} current to inactivate exponentially with a time constant (τ) of 277 ± 23 msec (data not shown), a value very similar to that obtained for the same channel subunits when transiently expressed in HEK 293 cells (17). In the remaining cells (9 of 44), currents inactivated with a significantly longer time course (1060 ± 146 msec; data not shown), indicating there may be some heterogeneity in channel properties from cell to cell. Because limiting of dilution is used to isolate the G1A1 cell line (see Materials and Methods), this heterogeneity may have arisen during subsequent propagation or may be some aspect of cell-to-cell variability. Third, both activation and steady state inactivation of inward Ba^{2+} currents were voltage dependent (Fig. 1A): the $V_{1/2}$ for activation was determined to be ~−8 mV under these recording conditions, and the $V_{1/2}$ for steady state inactivation was ~−70 mV. Finally, the pharmacology of the Ca^{2+} channels expressed by G1A1 cells was identical to that reported elsewhere for N-type Ca^{2+} channels. Channels were completely insensitive to inhibition by the dihydropyridine nifedipine (5 μM, n = 3; data not shown), reversibly and potently inhibited by Cd^{2+} ions (50 μM $CdCl_2$, 97% inhibition, n = 3; data not shown), and irreversibly inhibited by the peptide ω-conotoxin-GVIA (2 μM, 98% inhibition, n = 3; data not shown). Thus, the Ca^{2+} channels stably expressed by G1A1 cells possessed biophysical and pharmacological properties similar to those of both naturally occurring N-type Ca^{2+} channels and the same channel subunits when transiently expressed in HEK 293 cells.

Modulation by mGluRs. Because HEK 293 cells readily express G protein-coupled receptors that negatively couple to adenylyl cyclase (18), it is possible that transiently expressed rmGluRs, which are known to negatively modulate adenylyl cyclase (19, 20), might also couple to the human N-type Ca^{2+} channel present in G1A1 cells. Not surprisingly, 10 of 10 mock-transfected (expression vector without insert) G1A1 cells did not respond to the application of 100 μM L-glutamate (see Fig. 1B), demonstrating that HEK 293 cells do not express endogenous mGluRs capable of coupling to Ca^{2+} channels.

Conversely, when G1A1 cells were transfected with mammalian expression vectors containing cDNAs for two group II rmGluRs (rmGluR2 and rmGluR3), the application of 100 μM L-glutamate caused substantial inhibition of whole-cell Ba^{2+} currents in a significant number of cells (Figs. 1, C and D). Specifically, for all rmGluR2 transfection experiments, 138 of 350 cells tested responded to this saturating concentration of glutamate with 63 ± 2% (mean ± standard error) inhibition (median, 67%), whereas for all rmGluR3 transfections, 100 of 213 cells exhibited 44 ± 1% inhibition (median, 44%). Notably, rmGluR3 inhibited significantly less (p < 0.001, unpaired t test) of the whole-cell Ba^{2+} current than did rmGluR2. The apparent transfection efficiencies for rmGluR2 (138 of 350, or 39%) and rmGluR3 (100 of 213, or 47%) expression constructs are similar to that seen using a green fluorescent protein expression construct.[1]

For both receptors, the inhibition was characterized by slowing of the macroscopic current activation kinetics, exemplified by the apparent reduction in the amount of inhibition at later times during the depolarizing test pulse. For example, in the traces in Fig. 1, C and D, inhibition was 64% and 41% for rmGluR2 and rmGluR3, respectively, when measured 15 msec after the onset of the test pulse, whereas at 75 msec after the initiation of the test pulse, inhibition was reduced to 55% and 29%. Also, inhibition mediated by rmGluR2 and rmGluR3 was readily reversible and dose dependent and exhibited no apparent tachyphylaxis after repeated applications of maximally effective agonist concentrations (Fig. 1, E and F).

Modulation involves PTX-sensitive heterotrimeric G proteins. It was necessary to determine whether the G protein or proteins in G1A1 cells that mediate modulation of the Ca^{2+} channels by group II rmGluRs were sensitive to PTX. Using conventional PTX treatment paradigms (0.5 μg/ml, 12–18 hr; see Ref. 1) on two separate occasions (data not shown), no rmGluR2-expressing, PTX-treated G1A1 cells were detected (9 of 9 cells tested), whereas 8 of 16 control cells tested exhibited 65 ± 2% inhibition in response to saturating concentrations of mGluR agonists (either 100 μM L-glutamate or t-ACPD). Because relatively low numbers of cells actually express transfected rmGluRs and large numbers of cells would have to be tested to arrive at a convincing conclusion, a limited PTX treatment (0.5 μg/ml, 1.5–2 hr) of transfected cells was used to substantially reduce, but not entirely eliminate, PTX-sensitive $G_{i/o}$ subunit function. In two separate experiments (Fig. 2A), limited PTX treatment of rmGluR2-expressing G1A1 cells significantly (p < 0.01, unpaired two-tailed t test) reduced modulation of whole-cell Ba^{2+} currents from 66 ± 3% (n = 13) for untreated control cells to 28 ± 7% (n = 11) for the PTX-treated of dishes. Similarly, a limited PTX treatment of rmGluR3-expressing G1A1 cells (Fig. 2B) significantly reduced inhibition (p <

[1] B. A. McCool, unpublished observations.

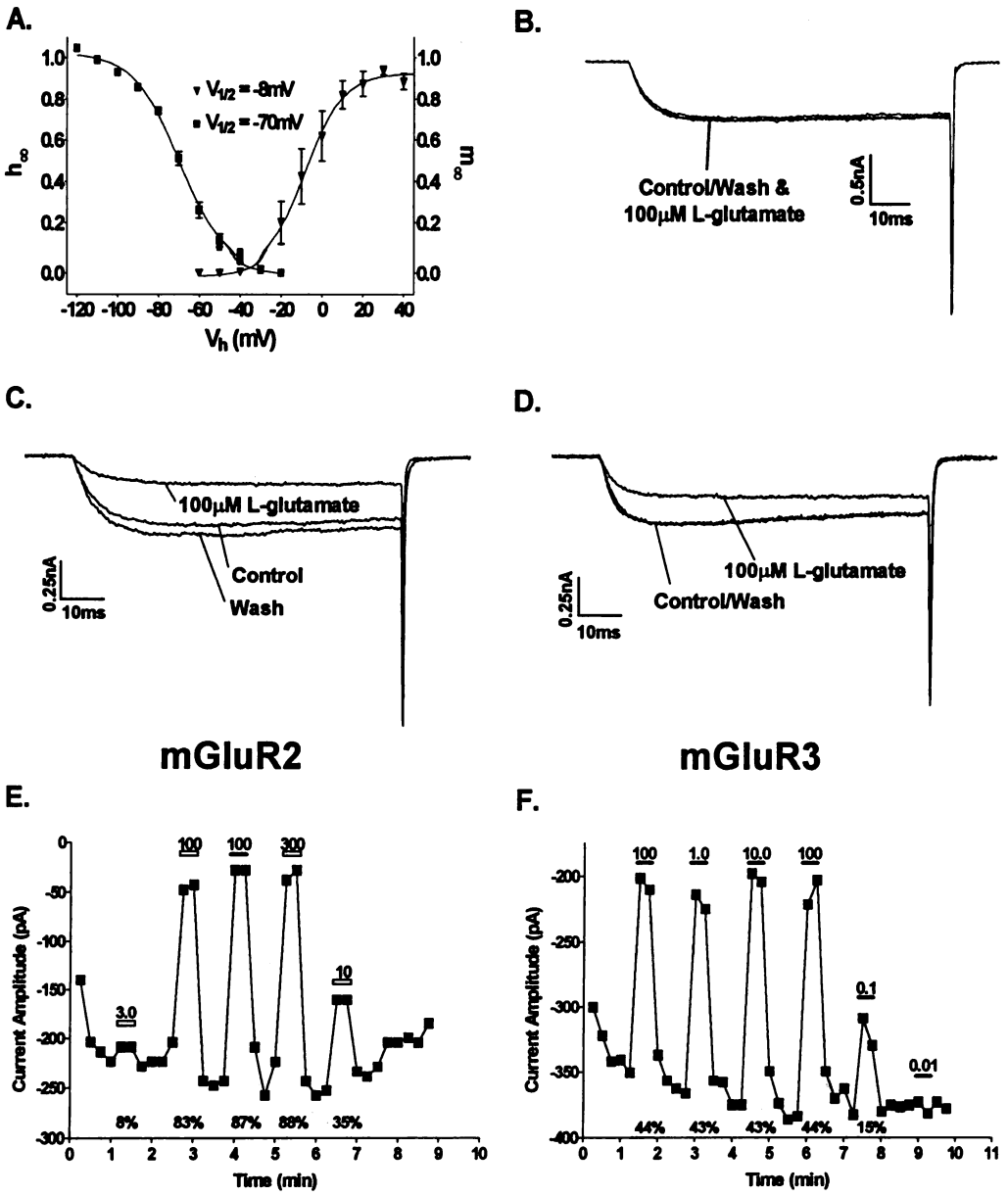

Fig. 1. Biophysical properties of the human α_{1B-1}, α_{2b}, and β_{1-3} Ca^{2+} channel subunits stably expressed by G1A1 cells and their modulation by group II rmGluRs. A, The voltage dependence of channel activation (m_∞, ▼) was estimated from extrapolated (first-order exponential fit) tail current amplitudes (sampled at 40 kHz) at different test potentials (holding potential −90 mV) using a protocol similar to that found in Williams *et al.* (17). Steady state channel inactivation (h_∞, ■) was measured from current amplitudes using a voltage protocol similar to one published elsewhere (10) except that the holding potential was −120 mV and test pulses were to −10 mV. The protocol was initiated at 1-min intervals. To find the half-maximal values, both activation and inactivation data sets were fit to sigmoidal Boltzmann functions: $h_\infty = \{1 + \exp[-(V - V_{½})/S]\}^{-1}$ and $m_\infty = \{1 + \exp[(V - V_{½})/S]\}^{-1}$, where V is membrane potential, $V_{½}$ is half-maximal value, and S is slope. B, G1A1 cells do not express endogenous mGluRs capable of modulating the stably expressed Ca^{2+} channels. Whole-cell Ba^{2+} currents in G1A1 cells were elicited by stepping from a holding potential (V_h) of −90 mV to a test potential (V_t) of −10 mV. For this and subsequent figures, currents were on-line leak-subtracted using a p/n protocol; only the corrected traces are shown. Ten of 10 G1A1 cells transfected with vector alone failed to respond to a saturating concentration of L-glutamate (100 μM). C and D, Inhibition of whole-cell Ba^{2+} currents in G1A1 cells by transfected rmGluR2 (A) and rmGluR3 (B) cDNAs was robust and reversible. Modulation was characterized by an apparent slowing of the macroscopic current activation kinetics (see Results). E and F, Inhibition by rmGluR2 (C) and rmGluR3 (D) is dose dependent (see also Fig. 6) and exhibits no obvious tachyphylaxis on repeated application of maximally effective agonist concentrations. t-ACPD (*open bars*) and L-glutamate (*closed bars*) were applied for ~30 sec. *Above the bars*, concentrations of agonist; *under the episodes*, percentage inhibition values for each application.

0.01, unpaired *t* test) from 34 ± 3% (n = 9) to 15 ± 4% (n = 9).

To address potential problems with dish-to-dish variability in transfection efficiency, expressing cells were first identified electrophysiologically and subsequently treated during the recording with low concentrations of the sulfhydryl-modifying agent NEM. At the concentrations used here, NEM selectively inactivates PTX-sensitive G protein α subunits (7, 21). Treatment with NEM (50 μM for 2 min) significantly ($p < 0.002$ in both cases, paired two-tailed Student's *t* test) reduced the amounts of inhibition in rmGluR2- or rmGluR3-expressing cells (Fig. 2, C and D) from 63 ± 9% to 3 ± 2% (n = 5) and from 51 ± 2% to −3 ± 2% (n = 5), respectively. Taken together, the results of the experiments with NEM and PTX

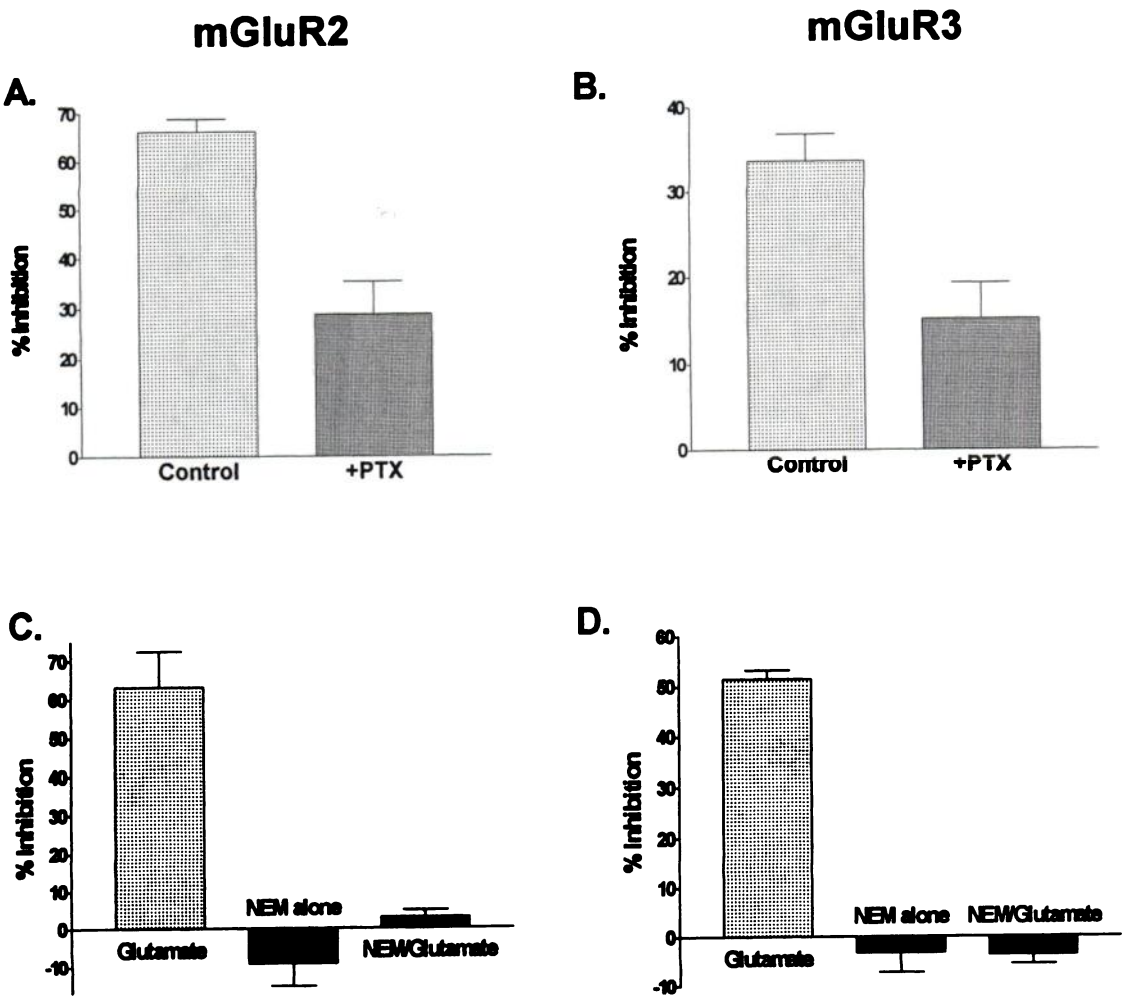

Fig. 2. Modulation of whole-cell Ba^{2+} currents by rmGluR2 and rmGluR3 was mediated by a PTX-sensitive G protein. A and B, In two separate experiments, limited PTX treatment (0.5 mg/ml for 1.5–2 hr) of rmGluR2-transfected G1A1 cells (A) significantly ($p < 0.01$; see Results) reduced Ca^{2+} channel modulation from 66 ± 3% ($n = 13$) in control dishes to 28 ± 7% ($n = 11$) in PTX-treated dishes. Likewise, for two separate rmGluR3 transfections (B), inhibition is significantly reduced ($p < 0.01$; see Results) from 34 ± 3% inhibition ($n = 9$) to 15 ± 4% ($n = 9$) by the same limited PTX treatment. In two separate experiments, overnight exposure to the same concentration of PTX prevented detection of any rmGluR2-expressing G1A1 cells (see Results). C and D, Both rmGluR2 (C) and rmGluR3 (D) modulation of Ba^{2+} currents was sensitive to NEM. rmGluR2- or rmGluR3-expressing G1A1 cells were first identified and then treated with NEM to inactivate PTX-sensitive G proteins during the course of the recording (see Results). Before NEM exposure, modulation was 63 ± 9% ($n = 5$) and 51 ± 2% ($n = 5$) for rmGluR2 and rmGluR3, respectively. A 2-min NEM treatment essentially removed all modulation, with inhibition being reduced to 3 ± 2% and −3 ± 2% in the same rmGluR2- and rmGluR3-expressing cells.

clearly indicate that modulation of the Ca^{2+} channels by group II rmGluRs was mediated through PTX-sensitive G protein α subunits.

Modulation is voltage dependent. Modulation by rmGluR2 and rmGluR3 was also characterized by voltage dependence. As with naturally occurring N-type Ca^{2+} channels (1, 22), the current-voltage relationships for the Ca^{2+} channels expressed by G1A1 cells in both the presence and absence of rmGluR modulation were bell shaped (Figs. 3, A and B). Furthermore, the inhibition mediated by rmGluR2 and rmGluR3 was substantially reduced as test potentials became increasingly depolarized (Figs. 3, C and D).

rmGluR modulation was also partially relieved (Fig. 4, A and B) by a depolarizing prepulse (23). In these traces, inhibition for the control pulse before the +80 mV depolarization (see Fig. 4) was 83% and 40% for rmGluR2 and rmGluR3, respectively, whereas inhibition for the test pulse after the +80 mV depolarization was reduced to 16% and 15% for rmGluR2 and rmGluR3, respectively.

Modulation is membrane delimited. The voltage dependence and depolarizing prepulse relief of Ca^{2+} channel inhibition for rmGluR2 and rmGluR3 mediated modulation were reminiscent of the well-documented membrane-delimited signal transduction pathway found for G protein-coupled receptors in peripheral neurons (reviewed in Ref. 24). To examine this further, the cell-attached macropatch configuration (15, 25) was used to determine whether inhibition by rmGluR2 and rmGluR3 in G1A1 cells was also membrane delimited. This approach was complicated by the finding that only a portion of the cells in a given transfection actually express rmGluRs. Also, the macroscopic, voltage-dependent inward Ba^{2+} currents were measured from the outside of the cell over a limited region of the cell surface and thus are quite small. Any contamination of the currents by endogenous delayed rectifier-type K^+ currents[2] might seriously compromise our ability to detect rmGluR-mediated modulation.

[2] B. A. McCool, unpublished observations.

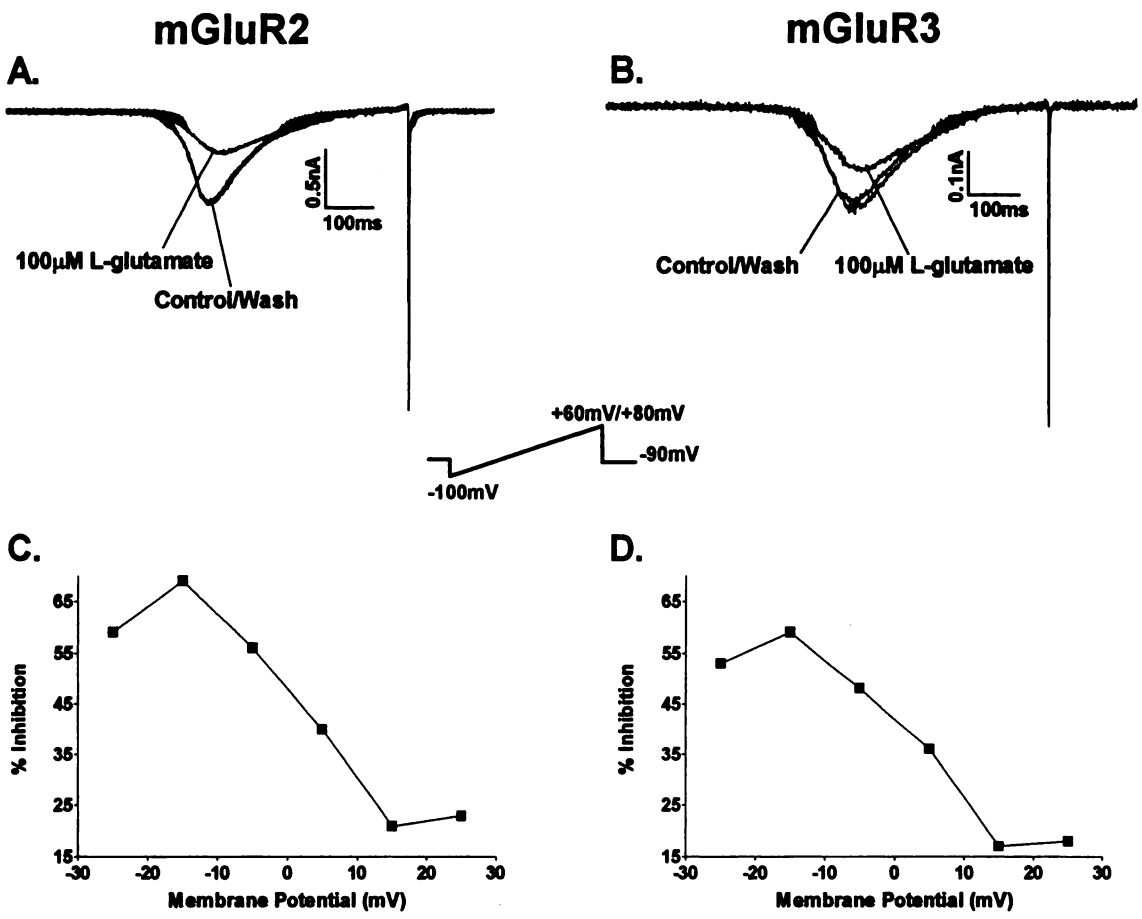

Fig. 3. rmGluR2 and rmGluR3 modulation of the G1A1 Ca^{2+} channel is voltage dependent. A and B, Current-voltage relationships were determined from a voltage-ramp procedure (protocol is indicated *under the traces*). The final voltage was +80 mV for the rmGluR2 trace (A) and +60 mV for the rmGluR3 trace (B). Inhibition by mGluR2 (*left*) and mGluR3 (*right*) did not change the shape of the current-voltage relationship. C and D, Percent inhibition was calculated from current amplitudes at specific membrane potentials in the traces in A and B and was seen to decrease as test potentials became increasingly depolarized.

Therefore, before recording in the cell-attached configuration, a given cell was examined with the whole-cell configuration to ensure that 1) this cell expressed functional mGluRs and 2) K^+ channel contributions to the macroscopic current were minimized by dialysis of the cell with the NMG/TEA internal (see Materials and Methods). After whole-cell recording, the pipette was carefully removed, and the rmGluR-expressing cell was recorded in the cell-attached configuration.

In the cell-attached configuration, depolarizing pulses to 0 mV from a holding potential of +90 mV (outside) caused inward Ba^{2+} currents that possess voltage/time-dependent inactivation characteristics similar to those of Ba^{2+} currents in whole-cell recordings (Fig. 5, A and B). Furthermore, current-voltage relationships for cell-attached currents were bell shaped with a peak around 0 mV (data not shown), indicating that there was no significant contribution to the recorded currents by K^+-selective conductances. The rmGluR2- and rmGluR3-expressing cells identified by whole-cell recording (Fig. 5, A and B, *insets*) did not respond to application of 100 μM L-glutamate when subsequently recorded in the cell-attached configuration (Fig. 5, A and B). Inhibition mediated by rmGluR2 and rmGluR3 in the whole-cell configuration was 66 ± 2% (n = 4) and 41 ± 4% (n = 4), respectively; conversely, the same rmGluR2- and rmGluR3-expressing cells recorded in the cell-attached configuration displayed −8 ± 3% and 3 ± 2% inhibition, respectively (Fig. 5, C and D). These results clearly indicate that modulation of Ca^{2+} channels by rmGluR2 and rmGluR3 in this HEK 293 expression system occurred via a membrane-delimited pathway.

rmGluR2 and rmGluR3 pharmacology. We have compared extensively the pharmacology of rmGluR2 and rmGluR3 (Fig. 6, A and B, and Table 1). Both receptors were insensitive to concentrations of group I-selective (10 μM quisqualate; data not shown) and group III-selective (L-SOP and L-AP4, 100 μM for each; data not shown) agonists, which are maximally efficacious for their respective group (reviewed in Ref. 2). We did not test concentrations of quisqualate >10 μM, although such concentrations have been shown to significantly activate rmGluR3 expressed in other systems (20). Both group II receptors were effectively activated by L-glutamate, t-ACPD, and the group II-selective agonists L-CCGI

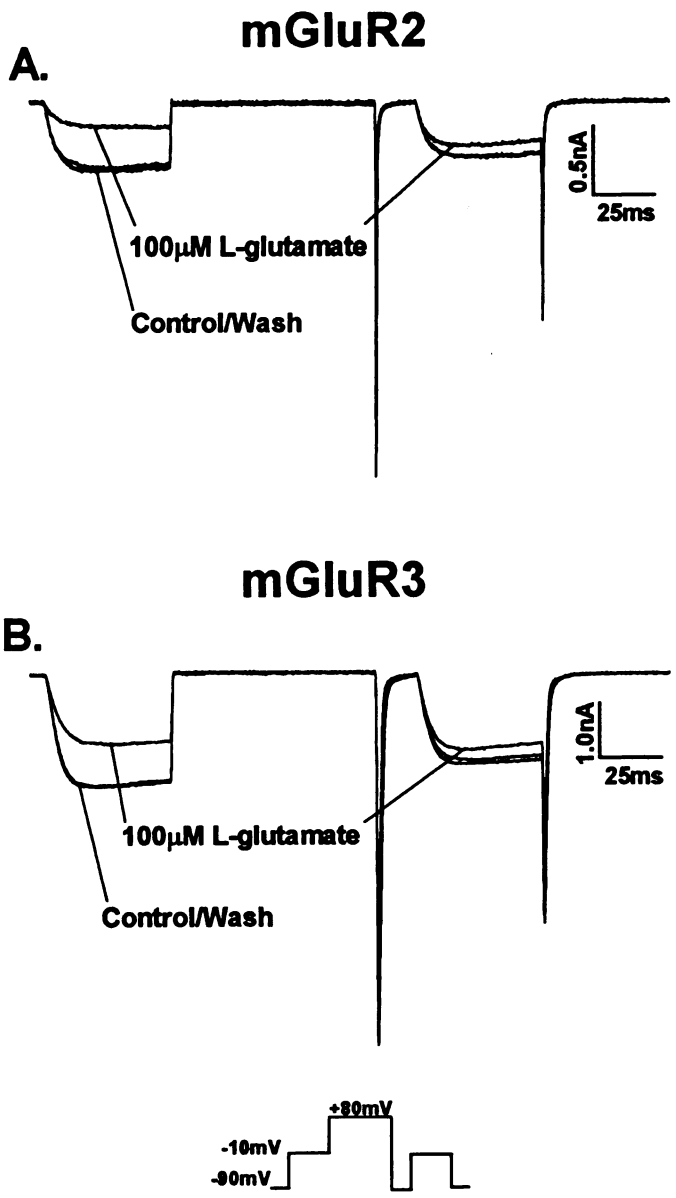

Fig. 4. A depolarizing prepulse partially relieved Ca^{2+} channel modulation by rmGluR2 and rmGluR3. A and B, *Under the current traces*, voltage protocol; parameters were identical to those published elsewhere (23). Percent inhibition, calculated from current amplitudes at 15 msec after the initiation of each test pulse (−10 mV), was 63% and 40% for the control pulse and decreased to 16% and 15% after the depolarizing prepulse for rmGluR2 and rmGluR3, respectively.

(26) and (*S*)-4C3HPG (27). It should be noted that both rmGluR2 and rmGluR3 were insensitive to the inactive enantiomer contained in t-ACPD, (1*R*,3*S*)-ACPD (100 μM; data not shown).

The relative potency of glutamate was similar for both receptors (Fig. 6 and Table 1). However, rmGluR2 was relatively more sensitive to the metabotropic agonist t-ACPD than was rmGluR3 (EC_{50} = 8.4 versus 32 μM, respectively). Surprisingly, L-CCGI and (*S*)-4C3HPG were markedly more potent for rmGluR2 (EC_{50} = 37 nM and 8.3 μM, respectively) than for rmGluR3 (EC_{50} = 0.5 and 89 μM). More important, L-CCGI clearly acted as a partial agonist for rmGluR3, with the highest concentration tested (30 μM) producing only ~80% of the inhibition of maximally effective concentrations of L-glutamate (Fig. 6B). For glutamate, t-ACPD, and (*S*)-4C3HPG, the Hill slope of the concentration-response curve of each receptor (Fig. 6, A and B) was 1.5–2.0, indicating some allosteric interaction or amplification in the signal transduction pathway. In addition, the Hill slope of the L-CCGI concentration-response curve for both rmGluR2 and rmGluR3 was substantially less than that for the other agonists, which is consistent with the notion that L-CCGI may be a partial agonist, at least for rmGluR3.

With regard to antagonists (Fig. 6, C and D), the IC_{50} was measured using an EC_{50} concentration of L-glutamate coapplied with varying concentrations of antagonists. Furthermore, to ensure that the antagonists were at equilibrium, the test concentration of antagonist was applied alone immediately before coapplication with an agonist. Both rmGluR2 and rmGluR3 were sensitive to the antagonists (+)-MCPG (27) and MCCG (28), while being insensitive to the group II mGluR-preferring antagonist M3C4HPG (29). MCCG seemed to be slightly more potent for rmGluR3 than for rmGluR2 (IC_{50} = 17 versus 24 μM, respectively), whereas MCPG was more potent for rmGluR2 than for rmGluR3 (IC_{50} = 190 versus 340 μM, respectively). Interestingly, the Hill slope for (+)-MCPG antagonism of rmGluR3 was noticeably less steep than that for either (+)-MCPG or MCCG at rmGluR2 or for MCCG antagonism at rmGluR3 (see Fig. 6, C and D, and Table 1).

Discussion

In the current study, we used a recombinant approach to determine the pharmacology and function of group II rmGluRs. To obtain physiologically relevant information, we also chose to examine group II rmGluR coupling to an effector specific to the central and peripheral nervous system, the voltage-gated N-type Ca^{2+} channel. To this end, we identify an HEK 293 cell line (G1A1) that stably expresses an ω-conotoxin GVIA-sensitive, N-type Ca^{2+} channel composed of the human α_{1B-1}, α_{2b}, and β_{1-3} subunits. The channels expressed by G1A1 cells possess biophysical and pharmacological properties that are essentially identical to those of naturally occurring N-type channels and of channels expressed from transient transfections of α_{1B-1}, α_{2b}, and β_{1-3} human Ca^{2+} channel subunits in HEK 293 cells (16).

We also showed that transiently expressed group II rmGluRs functionally couple to stably expressed human N-type Ca^{2+} channels in G1A1 cells using endogenous G protein-mediated, membrane-delimited signal transduction pathways. This coupling is dose and voltage dependent. The modulation by rmGluR2 and rmGluR3 is not induced by transient mGluR expression; somatostatin receptors expressed endogenously by G1A1 cells can also modulate the stably expressed N-type Ca^{2+} channel (30).[3] In any event, it can be recognized that the slowing of current activation kinetics by rmGluR2 and rmGluR3 modulation is not as pronounced in G1A1 cells as that seen for native G protein-coupled receptors in nonprimate central and peripheral neurons (6, 21–23). It is possible that this reduced kinetic slowing is a unique aspect of human N-type Ca^{2+} channel modulation or of the particular subunit combination (α_{1b}, α_2, β_{1-3}) used in these experiments. Alternatively, reduced ki-

[3] B. A. McCool and D. M. Lovinger, unpublished observations.

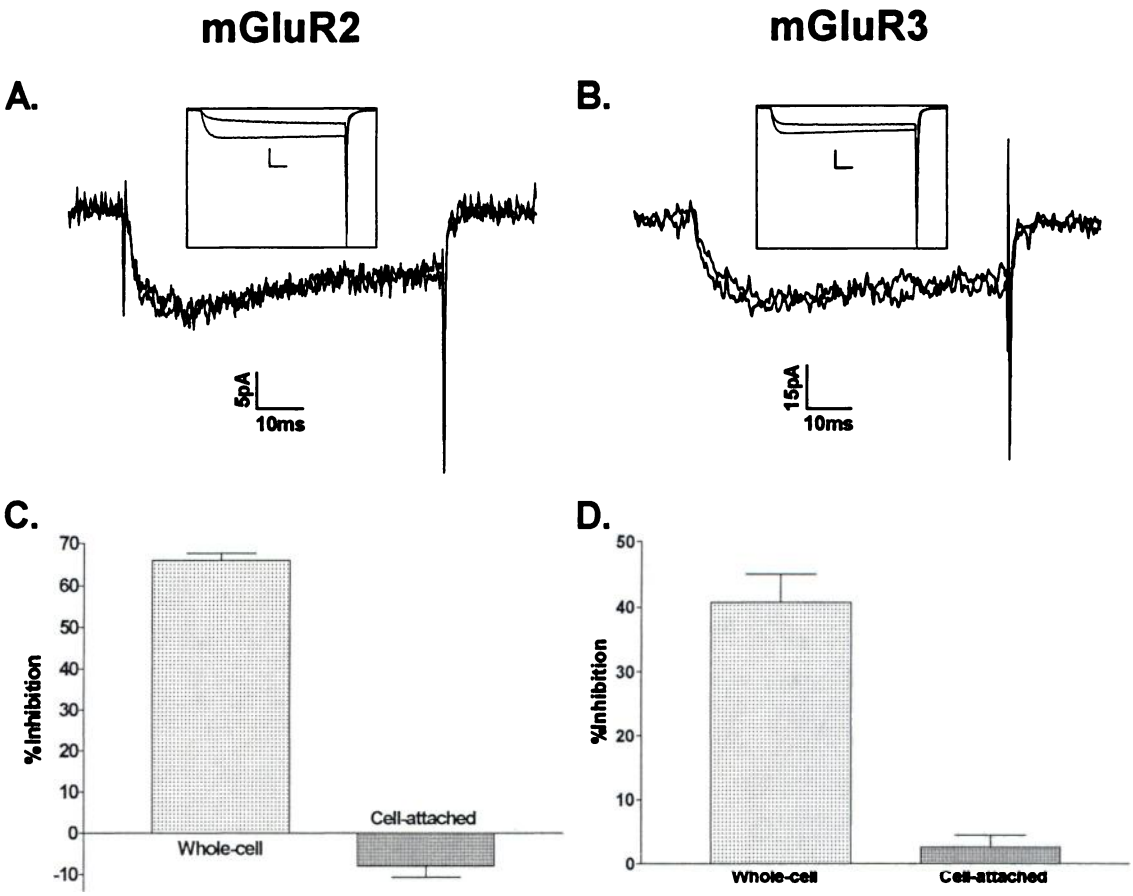

Fig. 5. Modulation of whole-cell Ba^{2+} currents in G1A1 cells by rmGluR2 and rmGluR3 is membrane delimited. A and B, Examples of currents recorded in the cell-attached configuration (see Results) from a rmGluR2- (A) and rmGluR3- (B) expressing cell that were unaffected (8% and −11% inhibition for rmGluR2 and rmGluR3, respectively) by agonist application (100 μM L-glutamate) outside the patch. However, currents recorded in the whole-cell configuration in these same cells (*inset*) were substantially inhibited (62% and 33% inhibition for rmGluR2 and rmGluR3, respectively) by application of the same concentration of agonist. *Calibration bars for whole-cell currents in inset*, 0.3 nA and 10 msec (A) and 1.0 nA and 10 msec (B). C and D, Summary of cell-attached experiments indicates that whole-cell currents are inhibited (100 μM L-glutamate; $n = 4$) by 66 ± 2% and 41 ± 4% for rmGluR2- and rmGluR3-expressing cells, respectively. Conversely, currents from the same cells exposed to the same concentration of agonist but recorded in the cell-attached configuration are inhibited −8 ± 3% and 3 ± 2% for rmGluR2 and rmGluR3, respectively.

netic slowing may be a manifestation of channel expression in HEK 293 cells. However, we observed in HEK 293 cells that transiently expressed "N-type" channels composed of rabbit α_{1b}, α_2, and β_3 subunits are robustly modulated by rmGluR2 with substantial kinetic slowing,[4] arguing more for some role of species differences or subunit composition than for the particular expression system used as a host for the channels.

It should be noted that both rmGluR2 and rmGluR3[5] can modulate N-type Ca^{2+} channels in a system in which G_o α subunits are believed to be the primary mediator (1). Although the parental HEK 293 cell line seems to express only G_i α subunits (31), we have not directly identified the α subunit responsible for the PTX-sensitive pathway in G1A1 cells. Thus, we cannot exclude the possibility that G1A1 cells also express G_o α subunits. Nevertheless, the possibility that G_i α subunit-containing heterotrimeric G proteins can modulate Ca^{2+} channels raises the interesting possibility that both α_i and α_o subunits are equally able to mediate various PTX-sensitive physiological responses. Also, the possible involvement of α_i-containing G proteins may support the emerging idea that G protein $\beta\gamma$ subunits, and not α subunits, are the active species in the receptor/Ca^{2+} channel signal transduction pathway (32, 33).

Of considerable interest to us was the apparent difference in agonist sensitivity for rmGluR2 and rmGluR3. The rank order of potency for L-glutamate and t-ACPD (i.e., glutamate > t-ACPD) is conserved between this system and other heterologous expression systems that use modulation of adenylyl cyclase activity as a measure of receptor function (19, 20). However, the absolute EC_{50} values for these agonists vary considerably, with glutamate in the G1A1 system (Fig. 7) being ~10-fold more potent than the adenylyl cyclase-derived values for both rmGluR2 and rmGluR3. The t-ACPD EC_{50} values for rmGluR2 are similar to those found elsewhere (8.4 μM in this report versus 5 μM in Ref. 19); conversely, the t-ACPD potency for rmGluR3 are ~4-fold lower in the G1A1 system (32 μM in this report versus 8 μM in Ref. 20). Hayashi *et al.* (26) reported that L-CCGI activates adenylyl cyclase-coupled rmGluR2 with an apparent potency of ~10-fold lower (EC_{50} = 300 nM) than the value we report for Ca^{2+} channel-coupled rmGluR2 (EC_{50} = 37 nM). Also, Thomsen *et al.* (34), using the adenylyl cyclase effector in BHK

[4] B. A. McCool, unpublished observations.
[5] Ikeda, S. (Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA), personal communication.

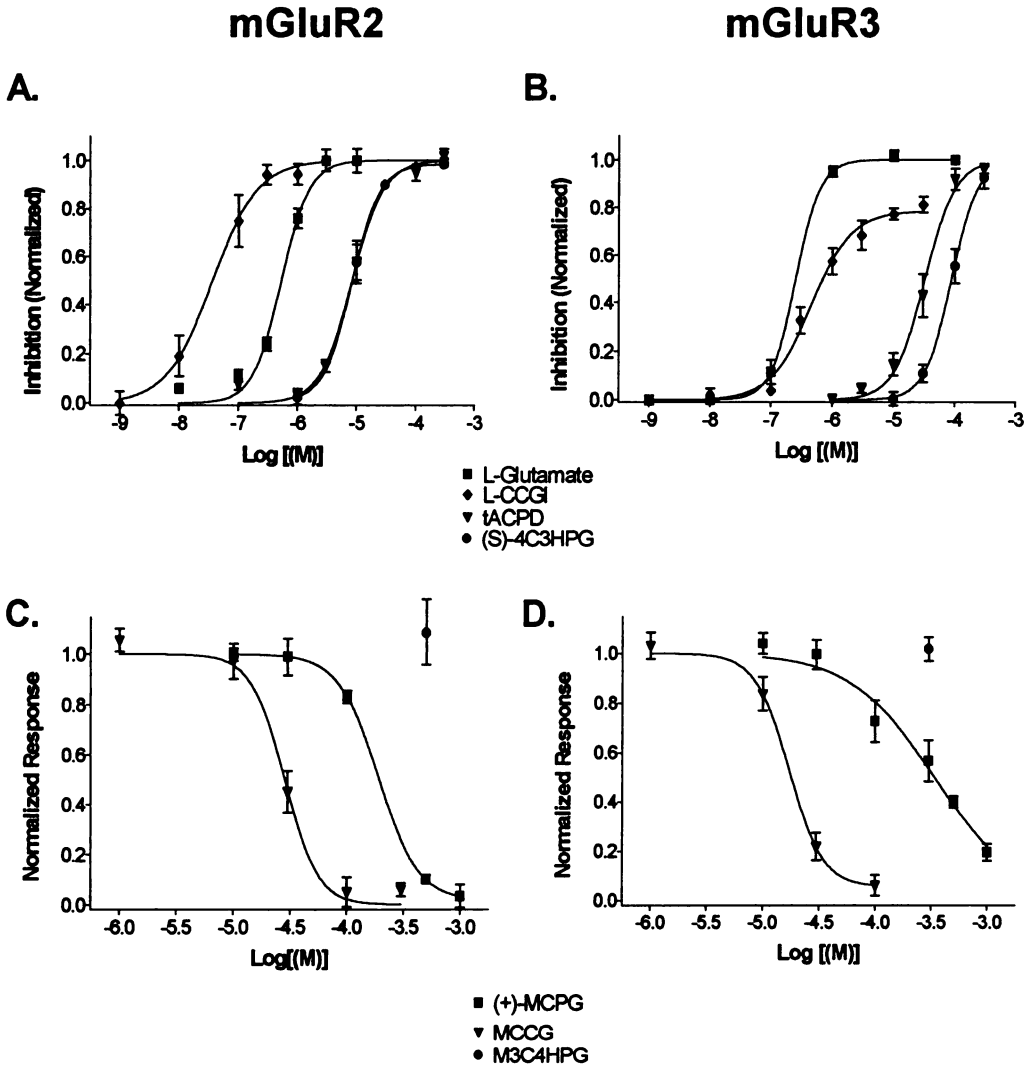

Fig. 6. rmGluR2 and rmGluR3 interact with agonists and antagonists in unique ways. A and B, Concentration-response curves for rmGluR2 and rmGluR3 indicate that the receptors were potently and reversibly activated by L-glutamate (■), t-ACPD (▼), and the group II-selective agonists L-CCGI (♦) and (*S*)-4C3HPG (●). EC_{50} values were computed from Hill plots of data normalized to percent inhibition by 100 μM L-glutamate and are shown with Hill slope values in Table 1. Both group II rmGluRs are insensitive to the group I-selective mGluR agonist quisqualate (10 μM), the group III-selective agonists L-AP4 (100 μM) and L-SOP (100 μM), and the inactive enantiomer present in t-ACPD, (1*R*,3*S*)-ACPD (100 μM; all not shown). C and D, Both rmGluR2 (C) and rmGluR3 (D) were sensitive to the competitive antagonists MCCG (▼) and (+)-MCPG (■). Conversely, both receptors were insensitive to the "group II-selective" antagonist M3C4HPG (●) (29). IC_{50} values were determined using an EC_{50} concentration of 0.5 and 0.3 μM L-glutamate (for rmGluR2 and rmGluR3, respectively) and are reported in Table 1.

cells, reported an mGluR2 EC_{50} value for (*R*,*S*)-4C3HPG of 48 μM. Considering that the (*S*)-isomer is the active species (27), this value is ~3-fold less potent than the value we report (EC_{50} = 8.3 μM). Because this is the first report that describes the actions of L-CCGI and (*S*)-4C3HPG on rmGluR3, we have no basis on which to compare the pharmacology of this receptor when expressed in various systems or coupled to different effectors.

We cannot rule out the possibility that the agonist pharmacology for rmGluR2 and rmGluR3 expressed in this system is substantially different from that of Ca^{2+} channel-coupled receptors found in native tissues. However, it is equally possible that relative differences in agonist sensitivity for receptors coupled to various effector systems in both native (i.e., neuronal) and heterologous settings are a reflection of the efficiency of coupling (35). In support of this, Ikeda *et al.* (1), who also assessed rmGluR2 function by modulation of N-type Ca^{2+} channels when heterologously expressed in sympathetic neurons, reported an EC_{50} value for glutamate that is lower (although only ~2-fold) than that found for this receptor when coupled to adenylyl cyclase (19). In addition, specific components of a given signal transduction pathway may significantly influence the absolute agonist sensitivity of a given G protein-coupled receptor. Indeed, Chabre *et al.* (36) showed that different G protein α subunits can significantly alter the apparent agonist EC_{50} (but not K_D) value of α_{2A} adrenergic receptors when these receptors are transiently coexpressed in HEK 293 cells with various G protein α subunits. These points may support the notion that agonist pharmacology in native and heterologous systems may not substantially differ provided the efficiency of coupling to a given effector and the identity of individual components in a given pathway are similar in both instances.

Although it is possible that system-specific phenomena such as differences in the number of spare receptors for rmGluR2- and rmGluR3-expressing cells may contribute to

TABLE 1

EC_{50}, IC_{50}, and slope values were estimated from Hill plots of data normalized to the percent inhibition by a maximal (agonists) or EC_{50} (antagonists) concentration of L-glutamate (see Fig 6 for the Hill plots)

Compound	mGluR2		mGluR3	
	EC_{50}	Hill slope	EC_{50}	Hill slope
L-Glutamate	0.5 μM	1.8	0.3 μM	2.2
L-CCG-I	37 nM	1.1	0.5 μM[a]	1.3[a]
t-ACPD	8.4 μM	1.6	33 μM	1.8
(S)-4C3HPG	8.3 μM	1.9	89 μM	2.0
	IC_{50}	Hill slope	IC_{50}	Hill slope
(+)-MCPG	190 μM	−2.5	340 μM	−1.2
MCCG	28 μM	−3.0	17 μM	−2.9
M3C4HPG	no effect[b]		no effect[b]	

[a] Partial agonist.
[b] The highest concentration tested was 300 μM (see text).

receptor sensitivity to various agonists, the effects of antagonists should be independent of such considerations. In this regard, both rmGluR2 and rmGluR3 are sensitive to (+)-MCPG and MCCG (27). Furthermore, the IC_{50} value for (+)-MCPG acting on rmGluR2 reported in this work is approximately half that reported for a racemic mixture used to antagonize mGluR2 inhibition of adenylyl cyclase (34). Surprisingly, the group II antagonist M3C4HPG proved to be ineffective against both of the cloned group II receptors. Because this antagonist was tested using L-CCGI as a agonist to inhibit forskolin-stimulated adenylyl cyclase activity in brain slices (29), it is certainly possible that L-glutamate-stimulated and/or Ca^{2+} channel-coupled mGluRs are only poorly antagonized by M3C4HPG. In support of this, agonist-specific effects on antagonist efficacy have been noted for group I receptors (37); however, these agonist-specific effects are only modest at best. The highest concentration of M3C4HPG used here was ~400-fold larger than the reported IC_{50} value for this compound in brain slices (29). Furthermore, preliminary indications are that M3C4HPG does not antagonize rmGluR3 coupled to N-type channels using an EC_{50} concentration of L-CCGI (data not shown). We can therefore suggest that M3C4HPG is not a antagonist for any of the cloned group II mGluRs.

The similar potency of L-glutamate along with the differential sensitivities of group II receptors to antagonism by (+)-MCPG and MCCG may actually indicate physical differences in the ways these receptors interact with agonists and antagonists. Because the amino acid sequences of the amino-terminal extracellular domains of rmGluR2 and rmGluR3 are strikingly similar (19) and this region plays the central role in agonist selectivity (38, 39), it is remarkable that these two receptors possess such unique pharmacologies. Nevertheless, our comparisons of primary amino acid sequences in the amino terminus of rmGluR2 and rmGluR3[6] indicate that the nonconserved regions occur in several areas believed to line the predicted binding pocket for glutamate (38). It is therefore possible that subtle differences in such regions will be further defined by standard mutagenesis approaches and eventually aid in the identification of truly receptor-specific agents.

[6] B. A. McCool, unpublished observations.

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