Agonist-dependent Signaling by Group I Metabotropic Glutamate Receptors Is Regulated by Association with Lipid Domains*

Received for publication, April 8, 2013, and in revised form, September 13, 2013. Published, JBC Papers in Press, September 17, 2013, DOI 10.1074/jbc.M113.475863

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Background: Lipid rafts regulate GPCR signaling.
Results: mGluR1 recruitment to lipid rafts is facilitated by a cholesterol recognition/interaction amino acid consensus motif and enhances agonist-dependent signaling.
Conclusion: Cholesterol within lipid rafts functions as an allosteric modulator of mGluR1 activity.
Significance: Cholesterol altering drugs may provide a means to modulate mGluR activity in neuropsychiatric conditions, including fragile X syndrome.

Group I metabotropic glutamate receptors (mGluRs), mGluR1 and mGluR5, play critical functions in forms of activity-dependent synaptic plasticity and synapse remodeling in physiological and pathological states. Importantly, in animal models of fragile X syndrome, group I mGluR activity is abnormally enhanced, a dysfunction that may partly underlie cognitive deficits in the condition. Lipid rafts are cholesterol- and sphingolipid-enriched membrane domains that are thought to form transient signaling platforms for ligand-activated receptors. Many G protein-coupled receptors, including group I mGluRs, are present in lipid rafts, but the mechanisms underlying recruitment to these membrane domains remain incompletely understood. Here, we show that mGluR1 recruitment to lipid rafts is enhanced by agonist binding and is supported at least in part by an intact cholesterol recognition/interaction amino acid consensus (CRAC) motif in the receptor. Substitutions of critical residues in the motif reduce mGluR1 association with lipid rafts and agonist-induced, mGluR1-dependent activation of extracellular-signal-activated kinase1/2 MAP kinase (ERK-MAPK). We find that alteration of membrane cholesterol content or perturbation of lipid rafts regulates agonist-dependent activation of ERK-MAPK by group I mGluRs, suggesting a potential function for cholesterol as a positive allosteric modulator of receptor function(s). Together, these findings suggest that drugs that alter membrane cholesterol levels or directed to the receptor-cholesterol interface could be employed to modulate abnormal group I mGluR activity in neuropsychiatric conditions, including fragile X syndrome.

Metabotropic glutamate receptors are G protein-coupled receptors (GPCRs)² prevalently and broadly expressed in the central nervous system where they regulate glutamatergic neurotransmission. mGluRs are encoded by a family of eight genes (GRM1–8) and categorized into three groups based on sequence homology, agonist selectivity, and signaling function (1). Group I mGluRs, mGluR1 and mGluR5, play critical functions in forms of activity-dependent synaptic plasticity, including hippocampal long term potentiation (2) and long term depression (3). Signaling by mGluR1 and mGluR5 contributes to synapse remodeling by inducing internalization of AMPA and NMDA receptors (4) and by stimulating rapid translation of mRNAs at synaptic sites (5). Altered mGluR1/5 activity is implicated in neurodevelopmental disorders including fragile X syndrome, the most common inherited cause of intellectual disability (6, 7). Importantly, in mice lacking the FMR1 gene encoding fragile X mental retardation protein, mGluR5 activity is abnormally enhanced, a dysfunction that may partly underlie cognitive deficits in fragile X syndrome (8, 9). Group I mGluRs preferentially couple to Go₃q, through which they engage the phospholipase C pathway and elicit phosphoinositide hydrolysis and intracellular calcium mobilization (1). Moreover, stimulation of group I mGluRs activates extracellular signal-activated kinase1/2 MAP kinase (ERK-MAPK) (10) and the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin (mTOR) pathway (11). The mechanisms underlying coordinated spatiotemporal regulation of group I mGluR-dependent activation of diverse signaling pathways remain as yet unclear.

Increasing evidence supports the existence of lateral heterogeneity in the plasma membrane, and many critical functions of lipid-protein interactions in cell physiology have been described (12). Lipid rafts are lipid domains of the plasma membrane enriched in cholesterol and sphingolipids; proteins are recruited to or segregated from lipid rafts depending on intrinsic affinity for the raft lipid environment (12). Proteins associ-
ated with lipid rafts are generally characterized by post-translational lipidation, including palmitoylation or the addition of glycosylphosphatidylinositol anchors (13). Transient, dynamic recruitment to lipid rafts was shown to promote assembly of active macromolecular signaling complexes, thereby contributing to the regulation of intracellular signaling efficiency and specificity (14, 15). GPCRs, including group I mGluRs (16, 17), are present in lipid rafts, but the mechanisms underlying association with these specialized membrane domains are incompletely understood.

Here, we investigated the molecular mechanisms underlying association of mGluR1 with lipid rafts and examined its impact on receptor signaling. Our findings indicate that mGluR1 association with lipid rafts is transiently increased by agonist binding and is dependent on membrane cholesterol content. We found that mGluR1 harbors a putative cholesterol recognition association/interaction consensus (CRAC) motif spanning the fifth transmembrane domain (TM5) and third intracellular loop (i3) of the receptor and that specific substitutions of critical residues in the motif impair mGluR1 association with lipid rafts. Furthermore, specific mutations in the putative CRAC motif appear to inhibit agonist-induced, mGluR1-dependent activation of ERK-MAPK without affecting mGluR1 constitutive activity. Consistent with a role of cholesterol in the regulation of mGluR1 signaling efficiency, increased cholesterol levels enhance mGluR1 response to stimulation by agonist, whereas acute cholesterol depletion inhibits agonist-induced, mGluR1-dependent ERK-MAPK activation. In neurons, inhibition of the mevalonate pathway with HMG-CoA reductase inhibitors (statins) similarly inhibits group I mGluR signaling and suggest that drugs that alter membrane cholesterol (e.g. statins, cyclodextrins) or targeting the receptor-cholesterol interface could be employed to modulate abnormal group I mGluR activity in neuropsychiatric conditions, including fragile X syndrome and autism.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Drugs, Reagents**—The following antibodies were used: goat anti-GAPDH antibody (GenScript, Piscataway, NJ), mouse monoclonal antibodies anti-mGluR1α, anti-flotilllin-1 (both from BD Biosciences), anti-myc tag (Cell Signaling Technology, Danvers, MA), anti-transferrin receptor 1 (Zymed Laboratories Inc., San Francisco, CA), anti-γ-tubulin, anti-α-tubulin (Sigma), and anti-pan actin (Lab Vision, Fremont, CA), and rabbit polyclonal antibodies anti-caveolin-1 and anti-Gαq/11 (both from Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-ERK1/2(Tyr-202/Tyr-204) and anti-ERK1/2 (both from Cell Signaling Technology), and anti-mGluR2 (Tocris Bioscience/R&D Systems, Minneapolis, MN). The group I mGluR-selective agonist (S)-3,5-dihydroxyphenylglycine (S-DHPG) (18), mGluR1-selective antagonists 7-hydroxymethyl-4-iminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt) (19), BAY 36-7620 (20), and tetrodotoxin were purchased from Tocris Bioscience/R&D Systems. l-Glutamic acid (sodium salt), methyl-β-cyclodextrin (mβCD), water-soluble cholesterol (cholesterol/mβCD mixture, ω-1:25 w/w ratio), lovastatin (mevinolin), simvastatin, and horseradish peroxidase-conjugated cholera toxin B were obtained from Sigma. Lovastatin and simvastatin were activated by dissolving in alkaline solution and heating at 50 °C (2 h) followed by pH neutralization.

**Preparation of Lipid Raft-rich Membranes from HEK293 Cells**—Membranes enriched with markers of lipid rafts were prepared according to the established protocols for the isolation of detergent-resistant membranes (DRMs) buoyant on isopycnic sucrose gradients (21, 22). Briefly, transfected cells were harvested in ice-cold PBS, washed 2 times with PBS, and solubilized in cold buffer of 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA (TNE buffer (pH 7.4)) containing 1% Triton X-100 (detergent:protein ratio ~ 3–2.5 to 1). After brief sonication, extracts were incubated on ice for 10 min, adjusted to 40% sucrose, transferred to centrifuge tubes, and overlaid with 6 ml of 30% sucrose and 3 ml of 5% sucrose. Samples were centrifuged at 34,000 rpm for 16 h with a SW40 Ti rotor (Beckman Coulter): 13 1-ml fractions were collected starting from the top of gradients. To identify lipid raft-rich membranes, equal volumes of each gradient fraction were analyzed by immunoblot to detect lipid and protein markers present in rafts (e.g. GM1, caveolin-1, flotilllin-1) and proteins excluded from rafts (transferrin receptor-1 (TfR1)). Lipid raft-rich membranes (DRM fractions 2–5) were defined by enrichment in GM1, caveolin-1, and/or flotilllin-1 and the absence of TfR1. Receptor abundance in DRMs relative to total was calculated from protein band densities: Σ (fractions 2–5)/Σ (fractions 1–13).

**Preparation of Lipid Raft-rich Membranes from Mouse Brain**—All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals by the United States Public Health Service. Adult wild type and caveolin-1 null (Cav1−/−) mice (The Jackson Laboratory, Bar Harbor, ME) were euthanized, and the forebrain was microdissected: ~300 mg of tissue were homogenized in ice-cold buffer of 10 mM Tris-HCl, 5 mM EDTA, 320 mM sucrose (pH 7.4) with protease inhibitors (Sigma). Homogenates were centrifuged at 800 × g for 10 min, and resulting supernatants were centrifuged at 10,000 × g for 15 min. Protein pellets were dissolved in ice-cold TNE buffer containing 1% Triton X-100, briefly sonicated, and incubated on ice for 10 min. Extracts were adjusted to 40% sucrose, transferred to centrifuge tubes, and fractionated on sucrose gradients as described for HEK293 cells.

**Cell Culture, Transfection, and Drug Treatments**—HEK293 cells (American Type Culture Collection; Manassas, VA) were cultured as described (17) and transfected by calcium phosphate precipitation or Lipofectamine 2000 (Invitrogen) by standard procedures. To reduce glutamate in the extracellular medium, cells were washed 2 times with Krebs buffer and incubated with 2 mM pyruvate and 2 units/ml glutamate-pyruvate transaminase (GPT; Roche Applied Science) for 2 h (23). To deplete membrane cholesterol, cells were incubated with 5 mM mβCD for 1 h at 37 °C in culture medium without antibiotics; to replenish membrane cholesterol after treatment with mβCD, cells were incubated with 2.5 mM cholesterol (water soluble form) for 1 h at 37 °C. To increase membrane cholesterol content, cells were incubated with 0.1 mM cholesterol for 2 h. Pri-
mary neurons were prepared from newborn Sprague-Dawley rats (Taconic, Hudson, NY) or FMR1 knock-out mouse pups (The Jackson Laboratory); for this, cells were dissociated from microdissected brain cortices by incubation with papain and gentle trituration and seeded onto poly-L-lysine-coated 10-mm plates or multi-well clusters. Neurons were maintained in DMEM supplemented with 2% (v/v) B27 (Invitrogen), 2 mM GlutaMax, and 5-fluoro-2’-deoxyuridine (Sigma) to prevent cell proliferation. Rat cortical neurons were used at 10–12 days in vitro for lipid raft preparation or ≥15 days in vitro for ERK assays. Mouse (≥23 days in vitro) or rat neurons were incubated for 3–4 days with lovastatin or simvastatin or vehicle; to measure ERK-MAPK activation, cells were rinsed with Krebs and incubated with [125I]iodoacetate (30 min) in conditioned medium to reduce spontaneous activity, washed 2 times with Krebs, and incubated with DHPG (50 μM) for indicated times. Specificity of acute lovastatin treatment was independently monitored in control neurons incubated for 24 h with 4 μM lovastatin together with 250 μM mevalonate (not illustrated).

Site-directed Mutagenesis—Single or double amino acid substitutions were introduced by site-directed mutagenesis with the QuikChange II XL system (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. Myc-tagged mGluR1, mGluR1 mutated in caveolin-1 binding domains (17), or carrying R775W or F781S amino acid substitutions (23) were previously described.

**ERK-MAPK Assays**—Protein quantification, separation by SDS-PAGE, transfer to nitrocellulose membranes, immunoblot, and detection by enhanced chemiluminescence were performed as previously described (17). Band densities were measured on digitized images using NIH Image/Image J software. The ganglioside GM1 was detected by dot-blot using cholera toxin B subunit conjugated to horseradish peroxidase as described (17). To label surface proteins with biotin, HEK293 cells were treated with GPT/pyruvate as described and quickly frozen at –80 °C. To prepare membranes, cells were thawed and suspended in an ice-cold buffer of 20 mM Hepes, 10 mM EDTA (pH 7.4) with protease inhibitors, disrupted with a Polytron homogenizer at 10,000 rpm for 10 s, and centrifuged at 48,000 × g for 30 min. Crude membrane pellets were washed with ice-cold buffer of 20 mM Hepes, 0.1 mM EDTA (pH 7.4), dissolved in the same buffer, divided into aliquots, and stored at –80 °C. Ligand binding assays were carried out according to conditions described in Mutel et al. (24). Briefly, membranes (~25 μg per assay) were thawed, washed once, resuspended in 20 mM Hepes (pH 7.4), and incubated for 1 h at room temperature with 2 mM MgCl2, 2 mM CaCl2, and 1, 5, 10, 30, 100 or 200 nM [3H]quisqualate; nonspecific binding was estimated by including 1 mM glutamate in control reactions. Labeled membranes were separated from unbound ligand by filtration through glass fiber filters using a cell harvester (Brandel, Gaithersburg, MD); filters were washed 3 times with a cold buffer of 20 mM Hepes, 2 mM MgCl2, 2 mM CaCl2, 0.3% polyethyleneimine (pH 7.4). Quisqualate affinity was calculated using GraphPad (GraphPad Software, La Jolla, CA).

Statistics—Data are expressed as the means and S.E.; statistical significance was determined by Student’s t test or ANOVA with p values < 0.05 considered significant.

**RESULTS**

**mGluR1 Association with Lipid Rafts Is Enhanced by Agonist Binding**—Lipid rafts were originally characterized and biochemically defined as a collection of buoyant membranes insoluble in cold non-ionic detergents and rich in cholesterol, glycosphingolipids, and glycolipids (25). Using these criteria, early studies provided evidence that proteins linked to glycosylphosphatidylinositol or modified by the addition of acyl or palmitoyl groups as well as some integral membrane proteins associated with lipid rafts. Purified membrane fractions of this kind do not equate to lipid microdomains in membranes of living cells, but they can provide the means to rapidly assess the presumptive affinity of proteins for different lipid environments (26). Group I mGluRs, mGluR1 and mGluR5, co-fractionate in the brain with membranes enriched in lipid raft-associated proteins and lipids (16, 17). However, only a subset of receptors is consistently recovered in lipid raft-rich membranes, suggesting that group I mGluRs may possess low intrinsic affinity for rafts or, alternatively, that the association may be transient and/or regulated by extrinsic factors. To begin addressing these questions, we used a model cell system to examine whether mGluR1 association with lipid raft-rich membranes is affected by exposure...
to glutamate, the receptor physiological ligand. To this end we expressed myc-mGluR1 in HEK293 cells and examined receptor abundance in buoyant DRMs, which are enriched in lipid raft-associated proteins (e.g. caveolin-1, flotillin-1) and lipids (e.g. GM1, cholesterol) but depleted of proteins normally excluded from rafts (e.g. TfR1) (17, 21). To measure the abundance of myc-mGluR1 in DRMs in the near absence of glutamate, transfected cells were incubated with GPT/pyruvate to rapidly convert free glutamate to L-ketoglutarate, a procedure that effectively reduces extracellular glutamate to trace levels (27). DRMs were isolated from cells treated with GPT/pyruvate alone (basal conditions) or subsequently incubated with 1 mM glutamate for 2, 5, and 20 min. Under basal conditions, ~5% of total mGluR1 was present in DRMs (Fig. 1, A and B). Application of glutamate increased the abundance of myc-mGluR1 in DRMs that was maximal at 5 min and declined to basal level upon prolonged exposure to agonist (Fig. 1, A and B). In contrast, application of glutamate did not significantly alter the abundance in DRMs of the lipid raft-associated proteins Gq/11 and caveolin-1 (Fig. 1C) or the distribution of transferrin receptor 1 (Fig. 1C), attesting to the specificity of the effect of glutamate on mGluR1. To test whether incubation with agonist produced a similar change in recruitment to lipid rafts of native group I mGluRs, we measured the abundance of mGluR5 in DRMs from primary rat cortical neurons under basal conditions (GPT/pyruvate) or in response to the application of

**FIGURE 1.** Group I mGluR association with lipid domains is enhanced by agonists. MGlur1 abundance in DRMs is increased by brief exposure to glutamate. A, representative immunoblots (IB) of extracts from transfected cells illustrating mGluR1 co-fractionation with DRMs in absence (basal) or presence of glutamate for different times; arrowheads indicate receptor monomers (~135 kDa) and dimers (~260 kDa). Numbers above gel lanes indicate gradient fractions (1, first fraction; 13, bottom gradient fraction): Input, total homogenate. B, quantification of mGluR1 abundance in DRMs in the absence (basal) or presence of glutamate for different times. MGlur1 abundance in DRMs (sum of band densities in fraction 2–5) is presented as the percentage of total receptor (sum of band densities in fractions 1–13) from images like those in A. Data are the means ± S.E.: basal, 4.58 ± 0.87% of total, n = 6; glutamate, 2 min, 8.68 ± 1.45%, n = 3; glutamate, 5 min, 20.73 ± 3.31%, n = 4; glutamate, 20 min, 10.48 ± 0.55%, n = 3. One-way ANOVA, Bonferroni post hoc; *, p < 0.05; **, p < 0.01; ***, p < 0.001. C, glutamate does not affect localization to DRMs of Gq/11, caveolin-1, or transferrin receptor 1. Representative immunoblots from extracts of myc-mGluR1 transfected cells corresponding to those in A were probed with indicated antibodies. Endogenous Gq/11 and caveolin-1 associate with DRMs with similar efficiency in the absence or presence of glutamate. Estimated Gq/11 abundance in DRMs versus total: basal, ~39%; glutamate, 5 min ~37%; glutamate, 20 min ~38%. D, MGlur5 abundance in neuronal DRMs is enhanced by exposure to DHPG. Rat cortical neurons were treated with GPT/pyruvate (1 h; basal) and stimulated with DHPG (50 μM, 5 min); representative immunoblots were probed with anti-mGlur5, anti-flotillin-1, and anti-transferrin-1 antibodies. MGlur5 abundance in DRMs was calculated as the percentage of total receptor and expressed as the ratio of DHPG versus basal; data are the means ± S.E.: 1.87 ± 0.115, n = 3, correlation coefficient r = 0.974 (ratio paired t test, significant, p > 0.05). Paired values in independent experiments were: control 7.09% versus DHPG 11.86%; control 49% versus DHPG 62.10%; control 3.7% versus DHPG 11.50%.
DHPG. In these experiments we chose to examine mGluR5 because it is present in DRMs from brain cortex and, unlike mGluR1, is highly abundant in cultured cortical neurons, thus facilitating detection (17). MGluR5 was present in DRMs under basal conditions, and its abundance in this membrane fraction appeared to be consistently increased upon brief application (5 min) of the group I mGluR selective agonist DHPG (Fig. 1).

Glutamate may regulate association with lipid rafts through activation of receptors or alternatively by transient stabilization of ligand-bound receptors in a conformational state with higher affinity for the raft lipid environment. To examine whether agonist-dependent enhancement of receptor abundance in DRMs depends on G protein activation, we used the non-competitive antagonist CPCCOEt (19) to inhibit receptor activity without occluding agonist binding. HEK293 cells expressing myc-mGluR1 were incubated with the group I mGluR selective agonist DHPG (50 μM, 5 min) or preincubated with CPCCOEt (100 μM, 20 min) and subsequently exposed to DHPG in the presence of antagonist. DRMs were isolated from drug-treated and control cells, and the relative abundance of mGluR1 in DRMs was evaluated by immunoblot. Similarly to glutamate, brief application of DHPG increased mGluR1 abundance in DRMs compared with basal (Fig. 2, A and B). In the presence of CPCCOEt, application of DHPG significantly increased mGluR1 abundance in DRMs (Fig. 2, A and B) albeit with partly reduced efficiency (% mGluR1 in DRM, CPCCOEt/DHPG versus DHPG, p < 0.01). Stimulation of group I mGluRs by agonists leads to activation of diverse intracellular signaling cascades including the ERK-MAPK pathway (10). To ensure that CPCCOEt effectively inhibited mGluR1 activity, we mea-

**FIGURE 2.** **MgLUr1 association with lipid domains is independent of G protein activation.** DHPG enhances mGluR1 abundance in DRMs in the presence of the non-competitive antagonist CPCCOEt. A, representative immunoblots (IB) of extracts from transfected cells probed with anti-mGluR1, -flotillin-1 (Flot-1), and -TfR1 antibodies illustrating mGluR1 co-fractionation in DRMs under basal conditions or after application of DHPG (5 min) in the absence or presence of CPCCOEt. *Input,* total homogenate; *arrowheads* indicate receptor monomers (~135 kDa) and dimers (~260 kDa). B, quantification of myc-mGluR1 abundance in DRMs from images like those in A are presented as the percentage of total receptor; data are the means ± S.E., n = 3; *, p < 0.05. ns, not significant. C, representative immunoblots, probed with anti-phospho-ERK1/2 (Thr-202/Tyr-204) and anti-ERK1/2 antibodies, of extracts from cells expressing myc-mGluR1 preincubated without or with CPCCOEt and stimulated with DHPG (5 min). D, quantification of ERK phosphorylation from experiments like those in C measured as the ratio of band densities for p-ERK2 versus ERK2; data are the means ± S.E., n = 4, one-way ANOVA with Bonferroni post test; *, p < 0.05; **, p < 0.01. E, representative immunoblot of extracts from transfected cells probed with anti-mGluR1 antibody illustrating co-fractionation in DRMs of the inactive mutant mGluR1F781S. Estimated mGluR1F781S abundance in DRMs versus total: basal, ~6%; DHPG (5 min), ~9%.

mGluR1 were incubated with the group I mGluR selective agonist DHPG (50 μM, 5 min) or preincubated with CPCCOEt (100 μM, 20 min) and subsequently exposed to DHPG in the presence of antagonist. DRMs were isolated from drug-treated and control cells, and the relative abundance of mGluR1 in DRMs was evaluated by immunoblot. Similarly to glutamate, brief application of DHPG increased mGluR1 abundance in DRMs compared with basal (Fig. 2, A and B). In the presence of CPCCOEt, application of DHPG significantly increased mGluR1 abundance in DRMs (Fig. 2, A and B) albeit with partly reduced efficiency (% mGluR1 in DRM, CPCCOEt/DHPG versus DHPG, p = 0.228). Stimulation of group I mGluRs by agonists leads to activation of diverse intracellular signaling cascades including the ERK-MAPK pathway (10). To ensure that CPCCOEt effectively inhibited mGluR1 activity, we mea-
sured its impact on DHPG-induced activation of ERK-MAPK. Cells expressing myc-mGlur1 were preincubated with vehicle or CPCCOEt and exposed for 5 min (time of peak ERK activation by mGlur1 in HEK293 cells (17)) to DHPG alone or in the continued presence of antagonist. As expected, DHPG induced a robust increase in ERK phosphorylation (Fig. 2, C and D) as determined by immunoblot with antibodies that recognize active/phosphorylated (Thr-202/Tyr-204) ERK1/2 and total ERK1/2 protein. In contrast, in the presence of the antagonist, DHPG failed to induce a significant increase in ERK phosphorylation (Fig. 2, C and D), indicating that CPCCOEt effectively inhibited mGlur1 activity under these experimental conditions. To determine whether a complete block of receptor activity could affect association with lipid rafts, we took advantage of an mGlur1 mutant (mGlur1F781S) carrying a single amino acid substitution in i3 that disrupts G protein coupling and blocks receptor activity (23). We found that when expressed in HEK293 cells, the relative abundance of mGlur1F781S in DRMs under basal conditions was similar to that of wild type mGlur1 (Fig. 2E; mGlur1F781S 6.8 ± 1.6% of total, n = 3, p > 0.05), and application of DHPG (5 min) produced a small increase (−9%) in raft association. Together, these findings indicate that mGlur1 association with lipid rafts does not require signaling via G proteins.

mGlur1 Association with Lipid Rafts Is Independent of Caveolin-1—Group I mGlurS interact with the scaffolding protein caveolin-1 (17, 28), which binds cholesterol and associates with caveolar rafts, one type of cholesterol-rich lipid domains (29, 30). In its oligomeric state, caveolin-1 forms membrane scaffolds (caveolar rafts) or becomes integrated in the coat of caveolae (30, 31). The lipid composition of caveolin scaffolds and caveolae is similar to lipid rafts, enriched in cholesterol and sphingolipids, and many proteins found in caveolae also associate with rafts (30, 32). Caveolin-1 interacts with an array of proteins (33) and was shown to mediate recruitment to caveolar rafts of some of its binding partners, including GPCRs (34). We examined whether caveolin-1 could play a role in promoting group I mGlur recruitment to lipid rafts using two different strategies. First, we took advantage of an mGlur1 mutant, mGlur1mut-i1/i3, in which caveolin-1 binding motifs in first and third intracellular loop of the receptor were disrupted, thereby preventing binding to caveolin-1 in vitro (17). Because loss of interaction with caveolin-1 might alter mGlur1 conformation and/or agonist affinity, we used membranes isolated from cells transfected with myc-tagged mGlur1 or mGlur1mut-i1/i3 to perform in vitro binding assays for quisqualate, a potent mGlur1 agonist (1). The measured affinity for quisqualate was similar for myc-mGlur1 and myc-mGlur1mut-i1/i3 (mGlur1 K_D 75.9 ± 31.6 nM, mGlur1mut-i1/i3 K_D 78.1 ± 26.6 nM, n = 6; Fig. 3, A and B) although lower than previously reported (24), presumably because of the presence of the tag close to agonist binding site. To examine the impact of caveolin-1 binding on receptor association with lipid rafts, HEK293 cells expressing myc-mGlur1mut-i1/i3 were incubated with GPT/pyruvate alone or challenged with 1 mM glutamate for 5 min; DRMs were prepared from control and glutamate-treated cells, and receptor localization was examined by immunoblot. Under basal conditions, only ~1% of myc-mGlur1mut-i1/i3 was present in DRMs (Fig. 3, C and D; 0.9 ± 0.5% of total, n = 3); application of glutamate increased myc-mGlur1mut-i1/i3 abundance in DRMs versus basal with efficiency comparable to wild type mGlur1 (myc-mGlur1 with glutamate, 4.7 ± 0.7-fold basal, n = 4, p < 0.01; myc-mGlur1mut-i1/i3 with glutamate, 7.6 ± 1.4-fold basal, n = 3, p < 0.05; fold basal, myc-mGlur1 versus myc-mGlur1mut-i1/i3 p = 0.137). Previous work showed that myc-mGlur1mut-i1/i3 mutants display reduced abundance at the plasma membrane (17), a property likely to underlie decreased association with DRMs under basal conditions. However, the finding that glutamate efficiently increased myc-mGlur1mut-i1/i3 abundance in DRMs suggests that caveolin-1 is not required for enhancement of mGlur1 recruitment to lipid domains by agonist(s). In a second approach, we examined whether native group I mGlurS associate with lipid rafts in the absence of caveolin-1. DRMs were isolated from the forebrain of wild type and caveolin-1 knock-out (Cav1−/−) mice, and lipid raft integrity and receptor localization was examined by immunoblot. DRMs from wild type and Cav1−/− mice were similarly enriched in GM1 (not illustrated) and flotillin-1 but depleted of transferrin receptor 1 (Fig. 3E), indicating that lipid domains are intact in absence of caveolin-1 (35). The relative abundance of native mGlur1 and mGlur5 appeared comparable in DRMs from Cav1−/− versus wild type mice (Fig. 3E), indicating that receptor localization to discrete membrane domains was not significantly affected. Together, these findings suggest that interaction with caveolin-1 is not required for group I mGlur recruitment to lipid rafts.

mGlur1 Association with Lipid Rafts Is Dependent on Membrane Cholesterol Content and a Putative CRAC Motif—Lipid rafts are rich in cholesterol, which affects lateral organization and fluidity of the plasma membrane (12, 22, 36). Extraction of membrane cholesterol alters the properties and composition of lipid rafts and caveolae but does not significantly affect other membrane microdomains (37). To gain insight on the mechanism(s) underlying mGlur1 recruitment to rafts, we tested whether receptor localization in DRMs is affected by alterations in cholesterol content. To acutely reduce membrane cholesterol, we used the non-ionic surfactant mβCD, which binds membrane cholesterol (38) and is commonly employed to perturb lipid raft composition. DRMs were isolated from myc-mGlur1-transfected cells that were incubated for 1 h with DMEM containing 5 mM mβCD (cholesterol depletion) or DMEM alone. Treatment with mβCD reduced cholesterol content in DRMs to ~12% of control cells (Fig. 4D). Under these conditions, mGlur1 and Ga_q/11, which is recruited to lipid rafts by means of its palmitate moieties (39), no longer co-fractionated with raft-enriched DRMs (Fig. 4A), unlike caveolin-1 that partly retained association with DRMs (Fig. 4C). To determine whether the effect of cholesterol depletion on mGlur1 localization was reversible, transfected cells were first treated with mβCD and then incubated for 1 h with 2.5 mM soluble cholesterol (cholesterol replenishment). Under these conditions, cholesterol content in DRMs was effectively restored compared with mβCD-treated cells (Fig. 4D), and both mGlur1 and Ga_q/11 were recovered in DRMs (Fig. 4, A and B).
The mechanisms underlying recruitment of integral membrane proteins to lipid rafts are not well understood, but recent studies have shown a critical function of covalently linked palmitate moieties in enhancing affinity for these membrane domains (13, 40). We searched the primary structure of mGluR1 by in silico analysis with publicly available software (CSS-Palm 2.0 (41)) and found no evidence of potential palmitoylation sites, in agreement with earlier studies (42). Interestingly, close inspection of mGluR1 amino acid sequence revealed the presence of a putative CRAC motif (43) spanning fifth transmembrane helix (TM5) and third intracellular loop (i3; Fig. 5A). A CRAC motif is defined by the consensus (L/V)X_{1–5}YX_{1–5}(R/K) (X, any amino acid) and is often present at junctions between membrane- and cytosol-exposed domains (43). Sequences potentially concordant with a putative CRAC motif were also detected in other mGluRs (Fig. 5B), including mGluR2, which shows enrichment in brain rafts (Fig. 5C). To determine whether the putative CRAC motif in mGluR1 plays a role in enhancing affinity for cholesterol-rich membrane domains, we used site-directed mutagenesis to substitute critical residues in the identified consensus sequence. Mutants with amino acid substitutions at either Leu-763 (mGluR1L763S), Tyr-769/Tyr-770 (mGluR1Y769A/Y770A), or Arg-775 (mGluR1R775G; mGluR1R775W (23)) were expressed in HEK293 cells, and their co-fractionation with DRMs was examined in the absence or presence of agonists (5 min). Under basal conditions, mGluR1L763S and mGluR1R775W were absent from DRMs (Fig. 5, D and G; mGluR1Y769A/Y770A, 0.45 ± 0.15% of total, n = 4; mGluR1R775G, 0.60 ± 0.41%, n = 3), whereas mGluR1Y769A/Y770A and mGluR1R775G partly co-fractionated with DRMs (Fig. 5, E and F), although less efficiently than the wild type receptor (mGluR1Y769A/Y770A, 3.46 ± 1.75% of total, n = 3; mGluR1R775G, 3.92 ± 1.33% of total, n = 4). In contrast to wild type mGluR1, all mutant receptors lacking intact CRAC consensus failed to associate with DRMs upon exposure to agonists (Fig. 5, D–G). We reasoned that inefficient recruitment to cho-

**FIGURE 3.** MGlur1 association with lipid domains is independent of caveolin-1. Glutamate enhances the association with DRMs of a mutant receptor (myc-mGluR1mut-i1/i3) lacking functional caveolin-1 binding domains. A and B, mutation of caveolin-1 binding motifs does not affect receptor affinity for agonist. Saturation isotherms of [3H]quisqualate binding to myc-mGluR1 (A) and myc-mGluR1-i1/i3 (B) are shown. C, representative immunoblots of extracts from transfected cells probed with anti-mGlur1 antibody illustrating mGluR1mut-i1/i3 co-fractionation in DRMs in absence (basal) or presence of glutamate applied for 5 min. Input, total homogenate. D, quantification of myc-mGluR1mut-i1/i3 co-fractionation in DRMs from images like those in C expressed as fold change in receptor abundance in DRMs in the presence of glutamate versus basal; data are the means ± S.E.; basal: 1.0 ± 0.5; glutamate, 7.6 ± 1.4-fold basal, n = 3; *, p < 0.05. -Fold change of wild type mGlur1 abundance in DRMs was calculated from experiments in Fig. 1 and is presented here for comparison. E, native mGluR1 and mGluR5 are present in lipid domains in the absence of caveolin-1. Representative immunoblots (IB) of DRMs isolated from wild type and caveolin-1 knock-out (Cav1−/−) mouse brain cortex probed with anti-mGluR1 and -mGluR5 antibodies are shown. Immunoblots with antibodies against flotillin-1 (Flot-1), a lipid raft marker, and TR1 excluded from rafts illustrate raft integrity in the absence of caveolin-1. Input, total cortical homogenate.
cholesterol-rich lipid domains could arise from altered expression of mutant receptors at the plasma membrane. To examine this possibility, HEK293 cells expressing either wild type or mutant receptors were labeled with cell-impermeable biotin at 8 °C; biotinylated surface proteins were recovered from lysates by precipitation with NeutrAvidin beads and examined together with input proteins by immunoblot with anti-mGluR1 antibodies. We found that the relative ratio of biotinylated versus total receptors did not significantly differ between wild type mGluR1 and mutants (Fig. 5, H and I), indicating that disruption of the CRAC consensus did not cause major deficits in receptor transport to and/or retention at the plasma membrane. Together, these findings suggest that mGluR1 associates with lipid rafts in a cholesterol-dependent manner and that a putative CRAC motif within the receptor contributes to enhance affinity for cholesterol-rich membrane domains.

Association with Lipid Rafts and Membrane Cholesterol Content Regulate Agonist-dependent, mGluR1-mediated Activation of ERK-MAPK—Activation of ERK-MAPK in response to certain stimuli and/or receptors is promoted by recruitment of activated signaling complexes to lipid rafts (44). We examined whether deficits in association with cholesterol-rich membrane domains arising from disruption of the CRAC consensus correlated to deficits in mGluR1 constitutive (27) or agonist-dependent activation of ERK-MAPK. To measure constitutive activity, HEK293 cells expressing either YFP (mock), wild type, or mutant receptors were incubated with vehicle or the inverse agonist BAY 36-7620 (10 μM, 30 min) and phosphorylated, and total ERK was measured by immunoblot as described. Similarly to wild type mGluR1, we found that in cells expressing mGluR1L763S, mGluR1Y769A/Y770A, or mGluR1R775W basal ERK phosphorylation was significantly enhanced compared with cells expressing YFP but restored to control levels by incubation with BAY 36-7620 (Fig. 6, A and B), indicating that mutant receptors retained constitutive activity. In contrast, application of DHPG (50 μM, 5 min) did not significantly increase ERK phosphorylation compared with basal levels (Fig. 6, A and C). However, unlike other mutants, mGluR1R775G, in which Arg-775 was replaced with glycine, displayed attenuated constitutive activity (Fig. 6, A and B) but retained the ability to activate

**FIGURE 4.** MGluR1 association with lipid domains is dependent on membrane cholesterol content. MGluR1 association with DRMs is abolished by cholesterol depletion and restored after cholesterol replenishment. A, representative immunoblots (IB) of DRMs prepared from transfected cells in which cholesterol was acutely removed from membranes by incubation with mβCD (depletion) or added back after depletion (replenishment); control cells were left untreated. Immunoblots were probed with antibodies against MGluR1, the lipid raft-associated protein Gq11, or TfR1, which is excluded from lipid rafts; Input, total homogenate. B, quantification of myc-MGluR1 co-fractionation in DRMs from images like those in A. Abundance in DRMs is presented as the percentage of total receptor; data are the means ± S.E.: control, 9.1 ± 1.4% of total, n = 4; cholesterol depletion, 1.0 ± 0.6% of total, n = 3; cholesterol replenishment, 29.6 ± 6.7% of total, n = 4; *, p < 0.05. Estimated Gq11 abundance in DRMs from images like those in A, means ± S.E.: control, 10.5 ± 4.4% of total; cholesterol depletion, none detected; cholesterol replenishment, 13.5 ± 3.2% of total. C, cholesterol depletion reduces but does not abolish association of endogenous caveolin-1 with DRMs prepared from cells as those in A; representative immunoblots were probed with anti-caveolin-1 antibody. Estimated caveolin-1 in DRMs was expressed as the percentage of total: control, 41%; cholesterol depletion, 23%; cholesterol replenishment, 48%. D, DRMs are rich in cholesterol and relatively depleted of proteins (protein content in DRM 4.6 ± 1% of total, n = 3); cholesterol can be rapidly and effectively removed from and restored to membranes by incubation with mβCD or a mβCD-cholesterol complex, respectively. Cholesterol concentration is in μg/ml in DRMs (fraction 3); data are the means ± S.E., n = 5 from two independent experiments.
ERK-MAPK in response to agonist (Fig. 6, A and C) and partial affinity for lipid domains under basal conditions. Whereas the bulky structure of tryptophan may induce a profound conformational change in the juxtamembrane region and mask the accessibility of the adjacent lysine (Lys-774), introduction of glycine could allow access to the adjacent positively charged Lys-774 residue that may compensate for Arg-775 structural role within the CRAC motif.

We reasoned that if the association with cholesterol-rich lipid domains is required for efficient activation of ERK-MAPK signaling by mGluR1, alterations in membrane cholesterol content could affect agonist-dependent mGluR1 activity, and we tested this possibility by three strategies. First, we examined the impact of acute cholesterol depletion on mGluR1-dependent activation of ERK-MAPK; for this, cells expressing mGluR1 were incubated with vehicle or mBCD (10 mM, 30 min) and...
exposed to 50 μM DHPG for 5 or 10 min, and ERK phosphorylation was measured by immunoblot. As expected, under control conditions DHPG induced a rapid and robust increase in ERK phosphorylation compared with basal (Fig. 7, A–C). In cells treated with mβCD, basal phosphorylated ERK was increased compared with control (Fig. 7, A–C), whereas DHPG-dependent ERK phosphorylation was significantly reduced (Fig. 7, A–C). Next, to assess the impact of elevated membrane cholesterol content, we incubated mGluR1-transfected cells in the absence or presence of soluble cholesterol (0.1 μM, 2 h), applied DHPG for 5 min at either submaximal or saturating concentration (2.5, 5, or 50 μM), and measured ERK activation. In control cells, 50 μM DHPG induced a robust increase in phosphorylated ERK compared with basal (Fig. 7, D and E), whereas application of 2.5 or 5 μM DHPG induced a small increase that did not reach statistical significance (Fig. 7, D and E; calculated EC50, mean ± S.E., 7.10 ± 2.10 μM (18)). In contrast, in cells that received supplemental cholesterol, DHPG induced robust ERK phosphorylation at submaximal agonist concentrations (Fig. 7, D and E; calculated EC50, mean ± S.E., 2.62 ± 0.61 μM). To test whether increased membrane cholesterol content could enhance agonist-dependent signaling by receptors carrying a mutation(s) in the CRAC motif, we measured ERK-MAPK activation by the mGluR1R775G mutant in response to DHPG (50 μM, 5 min) in cells incubated with or without supplemental cholesterol. As previously observed (Fig. 6, A and C), stimulation of mGluR1R775G did not result in increased ERK phosphorylation in control conditions; similarly, mGluR1R775G failed to activate ERK in response to DHPG in cells that received supplemental cholesterol (Fig. 7, F and G). Last, we examined whether inhibition of the mevalonate pathway by HMG-CoA reductase inhibitors (statins) could affect mGluR1-dependent ERK activation in neurons. Statins inhibit biosynthesis of cholesterol and isoprenoid intermediates that are added to signaling proteins and were shown to perturb the composition and properties of cholesterol-rich membrane domains (45, 46). We examined the impact of statins on group I mGluR signaling in primary rat cortical neurons that were incubated with either 1 μM lovastatin or 0.5 μM simvastatin or vehicle for ~3 days and exposed to DHPG (50 μM) for 5 or 10 min. In neurons treated with lovastatin, basal ERK2 phosphorylation appeared elevated compared with control, although the difference did not achieve statistical significance (lovastatin, 1.44 ± 0.37 of vehicle, n = 6, p = 0.321). In vehicle-treated neurons, application of DHPG increased ERK phosphorylation that was maximal after application of agonist for 10 min (Fig. 8, A and B). In contrast, in neurons treated with either lovastatin or simvastatin, application of DHPG failed to induce robust increase of ERK2 phosphorylation (Fig. 8, A and B). As proof of principle of the potential impact of statins on agonist-dependent group I mGluR signaling in pathological conditions, we examined the impact of statins on primary neurons from FMR1 knock-out mice, an animal model of fragile X syndrome, in which group I mGluR activity was shown to be abnormally enhanced and causally linked to fragile X syndrome pathophysiology (7, 8). In neurons incubated with lovastatin or simvastatin, basal ERK2 phosphorylation was increased compared with control (ratio of p-ERK2 versus ERK2, mean ± S.E.: vehicle 0.58 ± 0.073, n = 10, lovastatin, 0.88 ± 0.094, n = 10, simvastatin 0.91 ± 0.085, n = 11; control versus lovastatin or simvastatin, p < 0.05), whereas basal phosphorylated ERK1 was not significantly different between conditions (p > 0.05). Upon treatment with lovastatin, DHPG did not induce further ERK phosphorylation compared with basal (Fig. 8, C and D), whereas after treatment with simvastatin DHPG induced a small but not statistically significant increase in ERK phosphorylation. Col
lectively, these findings suggest that agonist-induced, mGluR1-mediated activation of the ERK-MAPK pathway is highly sensitive to membrane cholesterol content and correlated with the capacity of mGluR1 to associate with lipid rafts.

DISCUSSION

Although many GPCRs possess intrinsic capacity to associate with lipid rafts, the mechanisms by which this occurs remain little understood. Here, we investigated the extrinsic factors and structural properties that promote mGluR1 recruitment to lipid rafts and examined the impact of association with cholesterol-rich membrane domains on receptor function. We found that mGluR1 association with biochemically defined cholesterol-rich membranes is a transient, regulated process that is promoted by agonists and is sensitive to the relative abundance of cholesterol in the plasma membrane. We identified a putative CRAC motif in mGluR1 and provide evidence of its functional role in enhancing receptor affinity for cholesterol-rich membranes. We show that the efficiency of agonist-induced activation of ERK-MAPK by mGluR1 is impaired in mutant receptors carrying specific amino acid substitutions at critical sites of a putative CRAC motif and is regulated by changes in membrane cholesterol content and/or perturbation of lipid rafts.

Despite intense interest in the functions of lipid rafts in cell signaling and trafficking, their properties and physiological relevance have undergone intense scrutiny (47). Studies in model membranes documented the intrinsic capacity of raft lipids to form lateral assemblies (48), but the lack of morphological features hampered visualization in vivo. However, recent deployment of advanced imaging techniques has provided evidence for the existence of membrane domains in vivo (12, 37, 49). Lipid rafts are believed to form specialized, metastable nanodomains that can dynamically recruit or segregate membrane-associated proteins depending on the corresponding affinity for the raft proteolipid environment. As rafts are particularly enriched in signaling effectors (50), one proposed function is that they can form platforms for ligand-activated receptors linked to specific signaling cascades, for example the Gαq/11/ phospholipase C pathway (15, 29, 51). However, transient seg-
regation of effectors present in lipid rafts from receptors that possess low affinity for the raft environment can reduce signaling activity, as exemplified by inhibition of the G/GH9251s/adenylyl cyclase pathway by sequestration in lipid domains (29, 52, 53). The mechanisms and/or properties that drive GPCR recruitment to lipid rafts remain little understood. Post-translational modification by covalent attachment of palmitate was shown to be critical for association with raft membranes for several GPCRs (54, 55). However, not all palmitoylated receptors associate with lipid rafts, and GPCRs lacking palmitoylation sites, including mGluR1 (Ref. 42 and this study), show some degree of affinity for raft membranes. Collectively, these observations suggest that in addition to palmitoylation, other mechanisms and/or structural features participate in conferring affinity for lipid domains. One alternative mechanism is via interaction with scaffold proteins, such as caveolin-1, that reside in rafts. Caveolin-1 interacts with many GPCRs (30, 56) and promotes their coupling to Go alpha/g/adenylyl cyclase pathway by sequestration in lipid domains (29, 52, 53).

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Recent structural studies of class A GPCRs have revealed that several receptors, including the 2-adrenergic receptor (61, 62) and A2A adenosine receptor (63), co-crystallize with non-covalently bound cholesterol moieties. Interaction with two cholesterol molecules by the 2-adrenergic receptor occurs via a shallow groove formed by parts of TMI/II/III and IV, a structural feature conserved in many class A GPCRs (62). Cholesterol can also establish contacts with proteins via recognition of different motifs/domains, including CRAC motif (43), sterol-sensing

FIGURE 8. Treatment with HMG-CoA reductase inhibitors impairs agonist-dependent, mGluR1-mediated activation of ERK-MAPK in neurons. A, representative immunoblots (IB) probed with anti-phospho-ERK1/2(Thr-202/Tyr-204), anti-ERK1/2, and anti-7-tubulin of extracts from cortical neurons incubated with vehicle or the HMG-CoA reductase inhibitors lovastatin or simvastatin and stimulated with DHPG for indicated times. B, quantification of ERK2 phosphorylation from experiments like those in A; data are the mean ± S.E.; n = 6, **p < 0.05, one-way ANOVA. C, representative immunoblots probed with anti-phospho-ERK1/2, anti-7-tubulin, and anti-7-tubulin antibodies of extracts from FMR1 knock-out neurons incubated with vehicle, lovastatin, or simvastatin and stimulated with DHPG. D, quantification of ERK2 phosphorylation from experiments like those in C; data are the means ± S.E.; **p < 0.05, one-way ANOVA.
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domain (64), and glycine zippers (65). We identified a putative CRAC motif in mGluR1 on the basis of conformity to a consensus sequence and presence in juxtamembrane regions of the receptor. Occurrence of the CRAC motif(s) was demonstrated in other GPCRs, including serotonin 5-HT<sub>1A</sub> receptor (66) and CB1 cannabinoid receptor (67), although its role in facilitating recruitment to lipid domains was not extensively characterized. Our findings indicate that specific substitutions of critical residues in the CRAC motif affect mGluR1 association with cholesterol-rich membranes in the absence or presence of agonist, underscoring its potential function in cholesterol sensing and facilitation of receptor association with lipid rafts. The putative CRAC motif is partly located in TMV, a domain involved in binding of an mGluR1-selective allosteric potentiator (68), suggesting that amino acid residues within TMV may play a critical function in favoring defined conformational states of the receptor. Interestingly, recent molecular dynamic simulations have shown that helix 8 in the carboxyl-tail of mGluR2 folds into an amphipathic α-helix in the presence of high cholesterol concentration, an effect mediated by both direct and indirect actions of cholesterol on receptor and membranes, respectively (69). Together with our observations of association with DRMs by other mGluRs (Fig. 5C) and the potential occurrence of putative CRAC motifs in additional mGluRs (Fig. 5B), these findings suggest that affinity for cholesterol-rich membranes may be a shared property among the mGluR sub-family of class C GPCRs.

mGluRs are constitutive dimers with a large extracellular agonist binding site formed by a bilobate domain (1). Although mGluR monomers can couple to G proteins in response to positive allosteric modulators, the dimeric configuration is required for the ability of agonists to promote active versus inactive conformational states of the 7-TM region (70). Membrane cholesterol may affect GPCR stabilization in certain conformational states by means of direct interaction with receptors and/or by indirect effects on the properties of the lipid bilayer (71). Whereas cholesterol was shown to modify ligand binding affinity of some class A GPCRs, it may also act by stabilizing receptor dimers or the 7-TM region in active conformation favoring G protein coupling, two possibilities that are not mutually exclusive. DmGluRA, the Drosophila ortholog of vertebrate group II mGluRs (72), can be cross-linked to cholesterol (73) and associates with sterol-rich membranes that enhance its agonist affinity (73, 74), indicating that cholesterol may contribute to stabilization of the ligand-bound active conformation of DmGluRA, although the structural elements supporting the interaction and its functional impact were not examined.

An important issue that needs to be addressed to understand cholesterol-GPCR interaction, is to elucidate its biological significance in relation to receptor signaling. Group I mGluRs signal chiefly by coupling to G<sub>αq/11</sub>, thereby leading to phospholipase C activation, but they also activate the ERK-MAPK pathway by G protein-dependent and -independent mechanisms (75–77). Group I mGluR-dependent activation of ERK-MAPK is critical to important physiological brain functions including forms of synaptic plasticity in the hippocampus (57, 78). We show that several mutations introduced at critical residues in the CRAC motif do not affect mGluR1 capacity to activate ERK-MAPK in a constitutive manner, whereas they “blunt” mGluR1 activity in response to agonist. One potential interpretation is that in the absence of ligand(s), mGluR1 is in an active conformation that promotes constitutive signaling outside of raft membranes where cholesterol content is low, whereas an agonist-stabilized, active conformation is favored by association with lipid domains with high cholesterol content. Whether mGluR1 activates ERK-MAPK via a common mechanism in rafts versus non-rafts membranes remains to be determined. Constitutive activity of group I mGluRs was shown to depend on the composition of the receptor carboxyl-tail (27), but recent findings indicate that full constitutive activation can be induced by intersubunit cross-linking mutations in the cysteine-rich domain of the receptor by mechanisms involving conformational changes in the 7-TM region (79). Moreover, mutations at a single residue (Thr-764) in TMV of mGluR8 affect constitutive and/or agonist-dependent activity depending on the nature of the substitution (80).

Consistent with a role for lipid rafts in the regulation of GPCR signaling, we found that reducing membrane cholesterol inhibits agonist-induced mGluR1-dependent activation of ERK-MAPK, whereas augmenting membrane cholesterol content increases the efficiency of mGluR1 signaling in response to submaximal agonist concentration. A potential interpretation of these observations is that association with cholesterol-rich membranes stabilizes agonist-induced active state of the receptor at least in part via interaction of cholesterol with mGluR1. Association with lipid rafts was shown to play a critical role in regulating the capacity of GPCRs to activate ERK-MAPK in response to stimulation by certain agonists (44). For some GPCRs, including µ opioid receptor, recruitment to lipid rafts is required for activation by selected agonists (44), whereas for the β<sub>2</sub>-adrenergic receptor, ERK-MAPK activation requires receptor translocation out of raft membranes (81). Such differences may reflect different modalities of ERK activation including whether G protein-dependent and -independent mechanisms are engaged. Nevertheless, our findings suggest that membrane cholesterol acts as an efficient endogenous allosteric regulator of agonist-induced mGluR1 signaling. The contribution of membrane environment to regulation of mGluR1 signaling efficiency is particularly important in view of potential implications for treatment of neuropsychiatric conditions in which group I mGluR function is abnormal. In FMR1 knockout mice, an animal model of fragile X syndrome, abnormally enhanced group I mGluR activity has been linked to pathological manifestations of the condition including elevated long term depression, dysmorphic dendritic spines, and increased susceptibility to audiogenic seizures (7). Moreover, the absence of fragile X mental retardation protein results in overactivation of the ERK-MAPK pathway that can be corrected by mGluR5 inhibition (8). We find that, similarly to the effect of cholesterol depletion in HEK293 cells, in vitro administration of lovastatin or simvastatin to FMR1 knock-out neurons inhibits DHPG-induced activation of ERK-MAPK. Statins act at an early step in the mevalonate pathway and inhibit synthesis of cholesterol and isoprenoid intermediates farnesyl pyrophosphate and geranylgeranyl pyrophosphate that are post-translationally added
to signaling effectors. Given their impact on lipid synthesis and protein isoprenylation, statins were shown to perturb the composition and properties (i.e., lateral mobility) of lipid rafts. The finding that DHPG-dependent signaling in FMR1 null neurons is blunted by statins is consistent with a critical role of lipid rafts in supporting group I mGluR physiological function(s) and underscores the potential for alternative pharmacological strategies to normalize receptor activity in disease state. Interestingly, lovastatin applied in vitro was shown to reduce abnormally elevated protein synthesis and group I mGluR-dependent long term depression in hippocampal slices from FMR1 knockout mice, whereas administration in vivo ameliorated audiogenic seizures (82). Although the impact of lovastatin on receptor signaling was not tested, these important advancements are consistent with the overall hypothesis that lipid rafts and cholesterol act as endogenous allosteric modifiers of group I mGluR function(s) in vivo. Our findings provide evidence for a novel mechanism by which mGluR activity can be tuned by the membrane lipid environment and establish a molecular rationale for pharmacological interventions in neuropsychiatric conditions linked to abnormal function of group I mGlRs, such as fragile X syndrome and autism.

Acknowledgments—We thank Drs. Anne Etgen and Diane Lebexgue (Albert Einstein College of Medicine) for reagents and assistance with binding assays. We acknowledge support by the RFK-JDRC Core services (NICHD P30 HD071593).

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