Venus Fly Trap Domain of mGluR1 Functions as a Dominant Negative Against Group I mGluR Signaling

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Beqollari D, Kammermeier PJ. Venus fly trap domain of mGluR1 functions as a dominant negative against group I mGluR signaling. J Neurophysiol 104: 439–448, 2010. First published May 12, 2010; doi:10.1152/jn.00799.2009. Metabotropic glutamate receptors (mGluRs) form covalently linked homodimers and contain large, N-terminal extracellular ligand binding, “venus fly trap” (VFT) domains. These domains, when expressed separately, are secreted as disulfide linked dimers and can dimerize with full-length receptors. mGluR splice variants have been described that contain only this domain, but the consequences of their interaction on receptor signaling have not been explored. Here it is shown that an mGluR1 mutant containing only the VFT is retained on the cell surface when a full-length receptor is co-expressed. Further, when expressed in rat superior cervical ganglion (SCG) neurons and modulation of native calcium currents is used as an assay for receptor activity, the VFT acts as a dominant negative with respect to mGluR1 signaling. Although full-length mGluR1 and mGluR5 are not known to heterodimerize, the mGluR5 VFT partially occludes mGluR1 signaling and the mGluR1 VFT potently occludes mGluR5 signaling in SCG neurons. In addition, an mGluR1 point mutant, mGluR1 C140G, which cannot covalently dimerize, functions like the wild-type receptor when expressed alone. The C140G mutant is inhibited by the mGluR1 VFT construct but does not retain the mGluR1 VFT on the cell surface, suggesting that the loss of C140 renders the interaction reversible. Finally, a peptide designed to disrupt mGluR1 dimerization reduced signaling through the C140G mutant receptor, but only when applied intracellularly for several hours, indicating that loss of signaling requires disruption of dimerization prior to plasma membrane insertion.

INTRODUCTION

Metabotropic glutamate receptors (mGluRs) are class C G protein coupled receptors (GPCRs) with widespread distribution in the CNS where they regulate physiological processes such as synaptic plasticity (Bolshakov and Siegelbaum 1994; Neyman and Manahan-Vaughan 2008; Rouach and Nicoll 2003; Volk et al. 2007) and electrical excitability (Nistri et al. 2006) and play important roles in a variety of pathologies (Kew 2003; Volk et al. 2007) and electrical excitability (Nistri et al. 2006), that possess only the extracellular N-termini have and interacts with some small molecule allosteric modulators (Goudet et al. 2004; Knoflach et al. 2001). These domains are linked by a short extracellular cysteine-rich domain believed to play an important role in translating the conformational changes in the VFT that result from ligand binding into G protein activation in the heptahelical region (Rondard et al. 2006).

Like many GPCRs, mGluRs can dimerize (Robbins et al. 1999). In contrast to the class A GPCRs, however, the dimerization state of mGluRs is well established (Jingami et al. 2003; Kubo and Tateyama 2005), and the functional consequences of their dimerization are beginning to be elucidated (Brock et al. 2007; Goudet et al. 2005; Kammermeier and Yun 2005; Kniazeff et al. 2004). Unlike the closely related GABA<sub>B</sub> receptors, which form obligate heterodimers between GBR1 and GBR2 (Kaupmann et al. 1998; White et al. 1998), mGluRs form stable, covalently linked homodimers (Jingami et al. 2003; Kunishima et al. 2000; Tsuchiya et al. 2002). This dimerization appears to be essential, as the dimer molecules function as a receptor unit. For example, group I mGluRs (mGluR1 and 5) do not exhibit full activity unless bound by ligand in both dimer subunits (Kammermeier and Yun 2005; Kniazeff et al. 2004), and G protein activation seems to be mediated by only one subunit at a time (Goudet et al. 2005). In short, the currently accepted model seems to point to the dimeric mGluR as the indivisible basic receptor unit.

While there is no evidence for heterodimerization across the different members of the mGluR family (but see Goudet et al. 2005), less is known about the ability of different splice variants to dimerize with each other. For example, there is controversy about whether mGluR1a and mGluR1b can dimerize (Kumpost et al. 2008; Remelli et al. 2008; Robbins et al. 1999), but splice variants of mGluR5 do appear to cross dimerize (Remelli et al. 2008). Further, truncated splice variants of several mGluR subtypes including mGluR1, mGluR6, and mGluR8 (Malherbe et al. 1999; Valerio et al. 2001; Zhu et al. 1999), that possess only the extracellular N-termini have been reported. To date, the function of these splice variants is not known, but it is likely that at least some can form dimeric receptors with full-length mGluRs because similar recombinant constructs of mGluR1, mGluR3, and mGluR5 can clearly dimerize with their full-length counterparts (Bates et al. 2002; Robbins et al. 1999; Selkirk et al. 2002; Yao et al. 2004) and when expressed alone are translated and secreted as covalently linked dimers (Robbins et al. 1999; Selkirk et al. 2002; Yao et al. 2004).

Therefore the functional effects of the N-terminal domain of mGluR1 were examined when co-expressed with full-length mGluR1 and mGluR5, and with a point mutation of mGluR1 that expresses and functions like the wild type but does not form covalently linked dimers. It was determined that the
truncated receptor could occlude signaling of both mGluR1 and mGluR5 as a dominant negative subunit. Further, the mechanism of this occlusion of signaling was investigated to determine whether it acted by preventing the formation of full-length receptor dimers.

**Methods**

**Cell isolation, DNA injection, and plasmids**

A detailed description of the cell isolation and cDNA injection protocol can be found elsewhere (Ikeda 1997). Animal protocols were approved by the university committee on animal resources (UCAR). Briefly, both SCGs were removed from adult male Wistar rats (175–225 g) following CO2 euthanasia and decapitation and incubated in Earle’s balanced salt solution (InVitrogen, Life Technologies Carlsbad, CA) containing 0.6 mg/ml trypsin (Worthington Biochemicals, Freehold, NJ) and 0.8 mg/ml collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h at 35°C. Cells were transferred to minimum essential medium (InVitrogen/Gibco), plated on poly-L-lysine (Sigma Chemical, St. Louis, MO) coated 35 mm tissue culture dishes and incubated (95% air and 5% CO2; 100% humidity) at 37°C for 2–4 h before cDNA injection. Following injection, cells were incubated overnight at 37°C and patch clamp or immunofluorescence experiments were performed the following day.

Injection of cDNA was performed with an Eppendorf 5247 microinjection and InjectMan (Madison, WI) NI 2 micromanipulator 4–6 h following cell isolation. Injection electrodes were made with a Sutter (Novato, CA) P-97 horizontal electrode pulling using thin-walled, borosilicate glass (World Precision Instruments, Sarasota, FL). Plasmids were stored at −20°C as a 1 mg/ml stock solution in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). All mGluR1 and mGluR5 constructs were injected at 100–130 ng/μl (pCDNA3.1+; InVitrogen). The C140G mutant was produced using the QuikChange Site-Directed Mutagenesis strategy (Stratagene). All neurons were co-injected with “enhanced” green fluorescent protein cDNA (0.02 μg/μl; pEGFPN1; BD Biosciences-Clontech, Palo Alto, CA) for identification of successfully injected cells.

All constructs were sequence confirmed. PCR products were purified with Qiagen (Valencia, CA) silica membrane spin columns prior to restriction digestion and ligation. Plasmids were propagated in the Top10 Escherichia coli strain (InVitrogen) and midipreps were prepared using Qiagen anion exchange columns.

**Electrophysiology and data analysis**

Patch pipettes were made with a Sutter P-97 horizontal puller from 8250 glass (Garner Glass, Claremont, CA) and had resistances of 1–3 MΩ. Series resistances were 2–5 MΩ prior to electronic compensation of 80%. Whole cell patch-clamp recordings were made with an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA) or EPC-7 patch clamp amplifier (Heka Elektronik, Germany). Voltage protocol generation and data acquisition were performed using custom software (courtesy Stephen R. Ikeda, NIIAA, Rockville, MD) on a Macintosh G3 or G4 computer (Apple Computer, Cupertino, CA) with an InstroTech (Port Washington, NY; now Heka Elektronik) ITC-18 or ITC-16 data acquisition board. Currents were low-pass filtered at 3–5 kHz using the 4-pole Bessel filter in the patch clamp amplifiers, digitized at 2–5 kHz and stored on the computer for later analysis. Experiments were performed at 21–24°C (room temperature). Patch-clamp data analysis was performed using Igor Pro software (Wavemetrics, Lake Oswego, OR).

The external (bath) solution for patch-clamp recordings contained (in mM): 155 tris hydroxymethyl aminomethane, 20 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 glucose, 10 CaCl2, and 0.0003 tetrodotoxin (TTX), pH 7.4. The internal (pipette) solution contained (in mM) 120 N-methyl-D-glucamine (NMG) methanesulfonate, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 CaCl2, 4 MgATP, 0.3 Na,GTP, and 14 tris creatine phosphate, pH 7.2. 1-Glutamate (Sigma) was used as the agonist for mGluRs. All drugs and control solutions were applied to cells using a custom gravity-driven perfusion system positioned ~100 μm from the cell that allowed rapid solution exchange (≤250 ms). The degree of mGluR-mediated calcium current inhibition was calculated as the maximal inhibition of the current in the presence of drug compared with the last current measurement prior to application of the drug.

**Immunofluorescence and TIRF imaging**

To detect surface expression of the myc-mG1-NT construct, a Cy3-conjugated, mouse anti-myc, monoclonal antibody (Sigma) was applied to live cells at room temperature for 25 min at a 1:150 dilution. Cells were subsequently washed five times in phosphate buffered saline (PBS), and images were acquired from live cells. Fluorescence images and figures were constructed using Adobe Photoshop (Adobe Systems, San Jose, CA) and Canvas software (ACD Systems of America, British Columbia, Canada). Quantitative image analysis was performed using Igor Pro.

TIRF and mGluR1-GFP epifluorescence images were obtained on an Olympus IX71 inverted microscope using the total internal reflection fluorescence (TIRF) ×60, 1.45 NA objective. Images were acquired with a Cooke Sensicam QE digital cooled CCD camera (Cooke, Romulus, MI) using TillVision software (Till Photonics GmbH). All epifluorescence images described in Fig. 2C and images analyzed in Fig. 3C, as well as all TIRF images, were acquired using identical parameters to permit comparisons across cells. TIRF images were acquired with longer exposure times than epifluorescence (200 vs. 50 ms) to account for the lower light levels using the TIRF method. Fluorescence levels reported are average pixel intensities from a large rectangular region of interest within the cell, taken from 12 bit tiff images. Background levels were indistinguishable across images, so background subtraction was not reported, although subtraction did not alter the findings nor the interpretation (not shown).

**Results**

**Full-length mGluR1 retains a myc-tagged, N-terminal mGluR1 construct on the plasma membrane**

A deletion mutant consisting of the first 590 amino acids of (rat) mGluR1 and incorporating a myc-epitope tag after amino acid 33 was constructed. Similar mGluR1 and mGluR5 deletion constructs are translated and secreted as dimers in various expression systems (Robbins et al. 1999; Selkirk et al. 2002). To determine whether our construct, termed myc-mG1-NT, could be detected on the plasma membrane in the presence of full-length mGluR1, a Cy3-conjugated primary anti-myc antibody was used to detect cell surface myc expression in live rat sympathetic neurons from the superior cervical ganglion (SCG) under a variety of expression conditions (Fig. 1). When myc-mGluR1 (the full-length wild-type construct) was expressed alone (with GFP), substantial Cy3 labeling was detectable (Fig. 1A, top). As expected, expression of myc-mG1-NT or the untagged, full-length mGluR1 alone did not result in detectable Cy3 labeling (Fig. 1A, center). However, when myc-mG1-NT was co-expressed with the full-length mGluR1, Cy3 labeling was clearly evident (Fig. 1A, bottom), suggesting that the myc-tagged construct is retained on the plasma membrane due to its interaction with mGluR1.

Quantification of Cy3 fluorescence is shown in Fig. 1B. Signal was quantified by averaging Cy3 fluorescence intensity at the membrane measured from a line scan of each epifluorescence image. Peak GFP fluorescence is shown as a control.
for expression efficiency. While GFP fluorescence was similar in both groups, a strong Cy3 signal was detected in cells co-expressing myc-mGluR1-NT and WT mGluR1 but was undetectable in cells expressing myc-mG1-NT alone. SCG neurons co-expressing mGluR1 with myc-mG1-NT and those expressing myc-mG1-NT alone had Cy3 fluorescence intensities (in arbitrary fluorescence units) of 19 ± 4 (n = 9 cells) and 4 ± 0.4 (means ± SE; n = 13), respectively. GFP fluorescence from the same cells measured 51 ± 9 and 77 ± 17, respectively. These data are consistent with the conclusion that myc-mGluR1-NT expressed alone is secreted and that full-length mGluR1 retains myc-mGluR1-NT on the plasma membrane, presumably via dimerization (Robbins et al. 1999; Selkirk et al. 2002).

**Myc-mG1-NT functions as a dominant negative against mGluR1 signaling**

Although several reports have demonstrated that the N-terminal VFT domains of mGluR1 and mGluR5 can dimerize with their respective full-length receptors (Bates et al. 2002; Robbins et al. 1999; Selkirk et al. 2002), the effect of this interaction on receptor signaling has not been tested (but see Tiao et al. 2008). To address this question, full-length mGluR1 was expressed alone and with myc-mG1-NT in SCG neurons, and mGluR-mediated calcium current modulation was measured at several glutamate concentrations (Fig. 2). Consistent with previous reports (Kammermeier and Yun 2005), when mGluR1 was expressed alone, glutamate inhibited SCG calcium currents with an EC50 of 1–2 μM (Fig. 2, A and B) and a maximal inhibition (at 30 μM) of 48 ± 5% (n = 10). Surprisingly, when myc-mG1-NT was co-expressed, the maximal inhibition at 30 μM glutamate was reduced to only 13 ± 3% (n = 11). This residual effect could be interpreted as signaling through the small number of mGluR1 homodimers because if mGluR1 and myc-mG1-NT are expressed at similar levels and form “heterodimers” as well as homodimers, ~25% of expressed receptors are expected to be full-length mGluR1 homodimers.
As a negative control, mGluR1 was co-expressed with the analogous N-terminal venus fly trap domain of (rat) mGluR5 (amino acids 1–574), myc-mG5-NT. Because mGluR1 and mGluR5 are not known to heterodimerize, this construct was expected to be without effect on mGluR1 signaling. Surprisingly, a significant reduction of the maximal calcium current inhibition at 30 µM glutamate to 32 ± 5% (n = 12) was observed in co-expressing cells, suggesting that some interaction between mGluR1 and the VFT domain of mGluR5 can occur. These results would suggest that the 7 transmembrane domain of mGluRs can impose some negative influence on mGluR dimerization, perhaps imparting selectivity to dimerization within the mGluR family.

An alternate interpretation of these data are that the truncated mutants reduce mGluR1 expression to varying degrees. To rule out this possibility, the expression levels of a C-terminally GFP-tagged mGluR1 (mGluR1-GFP) were examined when expressed alone or with myc-mG1-NT (Fig. 2C). Cells were also injected with cDNA for a nuclear targeted dSRed (dSRed-nuc) to independently identify successfully injected neurons. Total mGluR1-GFP expression and plasma membrane expression were examined using epifluorescence and total internal reflection fluorescence microscopy (TIRF), respectively. TIRF is used to illuminate only those fluorescent molecules within ~100 Å of the glass cover slip (Toomre and Manstein 2001). Total mGluR1-GFP expression measured by epifluorescence was not detectably altered when myc-mG1-NT was co-expressed. Average fluorescence intensity measured from epifluorescence images was 735 ± 115 (arbitrary fluorescence units ± SE; n = 16) and 836 ± 135 (n = 12) in SCG neurons expressing mGluR1-GFP alone and mGluR1-GFP plus myc-mG1-NT, respectively. Surprisingly, plasma membrane expression of mGluR1-GFP, measured from TIRF images, appeared to be slightly enhanced when myc-mG1-NT was co-expressed. Average fluorescence intensity from TIRF images was 209 ± 27 (n = 16) in control and 337 ± 60 (n = 12) in the myc-mG1-NT co-expressing cells (Fig. 2C; the same cells were used for epifluorescence and TIRF imaging). Finally, to verify that the fluorescently tagged construct was inhibited by myc-mG1-NT in a manner similar to the wild-type mGluR1, mGluR1-GFP was expressed alone or with myc-mG1-NT in SCG neurons and the magnitude of calcium current inhibition in response to 100 µM glutamate application was monitored. Consistent with the results using the untagged mGluR1, mGluR1-GFP produced 27 ± 6% (n = 7) inhibition of the native calcium current when expressed alone but only 1 ± 3% (n = 6) when myc-mG1-NT was co-expressed using identical cDNA concentrations for intranuclear injection as in the fluorescence experiments described above (Fig. 2C).

While the apparent increase in plasma membrane receptor in the presence of myc-mG1-NT was unexpected and difficult to interpret, the data provide strong evidence that the dominant
negative actions of myc-mG1-NT do not result from loss of full-length receptor expression. Together, these data indicate that the N-terminal domain of mGluR1 can act as a strong dominant negative against mGluR1 signaling.

**Myc-mGluR1-NT also functions as a dominant negative against mGluR5 signaling**

To gain a better understanding of the mechanism of the dominant negative actions of the N-terminal VFT domain of mGluRs, the effects of these domains when co-expressed with mGluR5 were examined. A glutamate dose-response curve was generated in SCG neurons expressing mGluR5b (Fig. 3). The EC$_{50}$ for mGluR5b was similar to that for mGluR1 ($\sim 1 \mu$M), although the maximal calcium current inhibition was slightly smaller. Surprisingly, co-expression of the mGluR5 VFT domain, myc-mG5-NT, had only a moderate effect on the mGluR5b dose-response in SCG neurons, decreasing the calcium current inhibition at 100 µM glutamate by less than half from 38 ± 5 (n = 13) in cells expressing mGluR5b alone to 24 ± 4% (n = 7) when co-expressed with myc-mG5-NT. Further, co-expression of the mGluR1 VFT, myc-mG1-NT, appeared to have as strong a dominant negative effect on mGluR5 signaling as on mGluR1 (Fig. 3), reducing calcium current modulation via mGluR5b to 7 ± 2% (n = 8) at 100 µM glutamate. These results suggest that the N-terminal region of mGluR1 can function as a potent dominant negative against signaling of both mGluR1 and mGluR5, while the mGluR5 N-terminus only weakly inhibits signaling in both receptors. To test whether the differential effects of myc-mG1-NT and myc-mG5-NT resulted from differences in their expression levels, live SCG neurons expressing mGluR1 plus either myc-mG1-NT or myc-mG5-NT were labeled with Cy3-anti-myc antibody, as in Fig. 1. As controls, neurons expressing only mGluR1 or myc-mGluR1 were also labeled (Fig. 3C). While no Cy3 labeling was evident in mGluR1 cells (0.75 ± 0.5, n = 4), no difference was detectable between the positive controls (myc-mGluR1 alone: 6.6 ± 1.7, n = 5) and cells with mGluR1 plus either myc-mG1-NT (9.5 ± 2.5, n = 5) or myc-mG5-NT (11.8 ± 2.4, n = 9). Thus the weaker effect of myc-mG5-NT on mGluR1 and mGluR5 signaling is not likely to be the result of weaker expression. Therefore regions in the heptahelical domains of group I mGluRs must participate in selectivity of dimer formation among group I mGluRs. By contrast, co-expression of myc-mGluR1-NT did not alter calcium current modulation via 100 µM glutamate in mGluR2-expressing SCG neurons, nor via native α$_2$-adrenergic recep-

![Graph A](image1.png)

**Fig. 3.** myc-mG1-NT, but not myc-mG5-NT functions as a strong dominant negative against mGluR5 signaling. A: glutamate dose-response curves obtained in SCG neurons expressing mGluR5b alone (●), mGluR5b + myc-mG1-NT (□), and mGluR5b + myc-mG5-NT (Œ). Number of cells in each group shown in parentheses. B: bar graphs showing SCG calcium current inhibition in the absence (□) and presence (Œ) of myc-mG1-NT expression using as an agonist 100 µM glutamate in cells expressing mGluR2, and 10 µM norepinephrine, which activates a native adrenergic receptor, as indicated. C: average (±SE) Cy3 epifluorescence from cells labeled as in Fig. 1 with the indicated expression. Number of cells in each group is shown in parentheses.
tors following application of 10 μM norepinephrine (Fig. 3B). These data demonstrate that the dominant negative effect of the mGluR1 VFT exhibits selectivity in that only group I mGluRs appear to be affected.

Noncovalently dimerizing mGluR1 mutant is inhibited by myc-mG1-NT but does not retain it on the cell surface

Homodimerization of group I mGluRs is mediated in part by a covalent link between the C140 residues (C129 in mGluR5) in each dimer subunit (Ray and Hauschild 2000; Romano et al. 2001). However, mutation of this residue does not abolish dimerization because noncovalent dimers can form (Ray and Hauschild 2000; Romano et al. 1996, 2001). To determine whether the dominant negative actions of myc-mG1-NT require this covalent linkage, the truncated construct was co-expressed with the point mutant mGluR1 C140G in SCG neurons, and receptor-mediated modulation of calcium currents was examined. When expressed alone, mGluR1 C140G functioned like the wild-type receptor, with a similar maximal inhibition of 37 ± 8% (n = 9; not significantly different from the wild-type mGluR1; P = 0.23) at 30 μM glutamate, and an EC50 of about 3 μM (Fig. 4, A and B). Co-expression of myc-mG1-NT with mGluR1 C140G revealed that the deletion construct had as strong a dominant negative effect on the mutant receptor as on the wild-type. In cells co-expressing mGluR1 C140G and myc-mG1-NT, the maximal current inhibition at 100 μM glutamate was only 8 ± 2% (n = 7).

The strong inhibitory effect of myc-mG1-NT led to the expectation that like the wild-type mGluR1, the C140G mutant would likewise cause retention of myc-mG1-NT on the cell surface. However, no myc-mG1-NT plasma membrane retention was detectable when the construct was co-expressed with mGluR1 C140G (Fig. 4C). Using methodology identical to that described for Fig. 1, the presence of the myc epitope on the plasma membrane was probed with a Cy3 anti-myc antibody in live cells. While SCG neurons co-expressing mGluR1 and myc-mG1-NT yielded significantly greater plasma membrane Cy3 fluorescence than un.injected cells, those expressing mGluR1 C140G and myc-mG1-NT did not. Together these data suggest that the dominant negative effects of myc-mG1-NT may arise from the prevention of dimerization of mGluRs with the caveat that dimers must form before insertion into the plasma membrane. The lack of myc-mG1-NT on the cell surface also suggests that the interaction between mGluR1 C140G and myc-mG1-NT is reversible, but the lack of signaling under these conditions suggests that mGluR1 C140G dimerization is not initiated on the plasma membrane or occurs there very slowly.

Selective disruption of mGluR1 C140G signaling with a dimer interface peptide

A plausible explanation of the data in the preceding text may be that the myc-mG1-NT construct occludes mGluR function by preventing dimerization of full-length receptors. This possibility was further probed by testing whether a 20 amino acid
peptide corresponding to the helical mGluR1 dimer interface (“DI-pep”; Fig. 5A) could disrupt mGluR function (Sato et al. 2003), possibly by preventing dimerization.

SCG neurons were initially isolated and double injected: intranuclearly and cytoplasmically, using a micropipette containing 100 ng/μl cDNA (encoding mGluR1) and 10 μM DI-pep, or containing cDNA alone as a negative control. For these mGluR1-expressing SCG neurons, no difference in mGluR1-mediated calcium current modulation was detectable. Average inhibition in control and peptide injected, mGluR1-expressing cells was 55 ± 10% (n = 3) and 58 ± 1% (n = 4), respectively (Fig. 5B). By contrast, when identical experiments were performed with mGluR1 C140G expression, the magnitude of calcium current inhibition was significantly reduced from 56 ± 7% (n = 5) in control to 30 ± 10% (n = 5) in peptide injected cells (Fig. 5B). These data are consistent with the interpretation that disruption of full-length mGluR dimerization can disrupt signaling but that the covalently linked wild-type receptors are sufficiently stable that the DI-pep is ineffective. It is possible that unlike the peptide, the myc-mG1-NT has effective dominant negative activity against the wild-type receptor because it contains the C140 residue, which may permit covalent dimerization with the full-length mGluR1.

To further characterize the activity of the DI-pep, glutamate-mediated calcium current modulation was examined in SCG neurons expressing either mGluR1 or mGluR1 C140G before and after acute application of 10 μM DI-pep to the bath for 1 min. Surprisingly, the magnitude of calcium current inhibition by neither mGluR1 nor mGluR1 C140G was detectably altered by acute peptide application in this way. Calcium currents were inhibited before and after DI-pep application in mGluR1 expressing cells by 55 ± 10 and 52 ± 11% (n = 3), respectively. Calcium currents in cells expressing mGluR1 C140G were inhibited 55 ± 7 and 51 ± 9% (n = 5) before and after acute DI-pep application, respectively. In addition, 1–2 h bath application of 10 μM DI-pep prior to recording was likewise ineffective at reducing mGluR1 C140G activity (Fig. 5C). Peptide-treated mGluR1 C140G-expressing cells showed calcium current inhibition of 42 ± 6% (n = 9) in paired mGluR1 C140G-expressing, untreated control cells, 37 ± 7% (n = 8) in cells pretreated for 1 h, and 39 ± 5% (n = 9) in those pretreated for 2 h. Figure 5C shows the combined data for all the bath applied, C140G experiments, pooling the control cells from all of these experiments. Note that while there appears to be a small reduction in inhibition after 1–2 h in the peptide, this difference was not statistically significant. Further, when these data are compared with the control cells recorded on the same days (as reported in the preceding text), no reduction is evident. Together these data strengthen the conclusion that mGluR function is occluded when full-length dimerization is disrupted, but that dimerization is fairly stable once the receptors are inserted in the plasma membrane, even for the C140G mutant receptor. However, if dimerization can be prevented before plasma membrane insertion, perhaps at the time of translation, such as when myc-mG1-NT is co-expressed or co-injected with mGluR1.

**FIG. 5.** Intracellular, but not bath-application of the dimer interface peptide, DI-pep reduces signaling of mGluR1 C140G. A, left: graphical representation of the structure of the N-terminal, VFD domain of mGluR1. Separate dimer subunits are shown in blue and pink. The yellow highlighted residues illustrate the residues of the DI-pep, showing its close proximity to the opposing dimer subunit. Right: sequence of DI-pep and graphical representation in isolation. B: average (±SE) calcium current modulation by 100 μM glutamate in SCG neurons expressing mGluR1 or mGluR1 C140G, as indicated, untreated (●) or preinjected cytoplasmically with 10 μM DI-pep (■), *, a statistically significant difference from control (P ≤ 0.05). C: average (±SE) calcium current modulation by 100 μM glutamate in SCG neurons expressing mGluR1 C140G, untreated or following 2 h of bath applied, 10 μM DI-pep. Number of cells in each group shown in parentheses.
when DI-pep is present intracellularly in the presence of the C140G mutant receptor, full-length dimers may fail to form and receptor signaling can be disrupted.

**DISCUSSION**

The data in the preceding text demonstrate that an N-terminal mGluR1 construct, similar to a truncated splice variant (Zhu et al. 1999), is retained on the plasma membrane when co-expressed with the full-length mGluR1 in rat sympathetic neurons. Further, co-expression of the recombinant mGluM1-NT construct strongly inhibits signaling of mGluR1, measured as the magnitude of mGluR-mediated calcium current modulation in rat SCG neurons. The remaining signal observed is consistent with the expected small number of full-length mGluR1 homodimers when both constructs are expressed, perhaps indicating that the mGluR1/mG1-NT dimers entirely lack the ability to signal. When the N-terminal VFT region of mGluR5 was expressed with mGluR1 as a negative control, some inhibition of mGluR1 signaling was observed, but much less than with myc-mG1-NT, even though myc-mGluR1-NT and myc-mG5-NT appeared to be expressed at roughly equal levels (Fig. 3C). It is difficult to interpret how equal levels of myc-mGluR1-NT and myc-mG5-NT on the cell surface could produce different degrees of signal occlusion if the mechanism relies on disruption of dimer formation. However, the immunofluorescence method used to measure the surface myc-epitope is unlikely to be sensitive enough to tease out subtle differences in expression levels. Thus it may be prudent to interpret these data as something of a digital signal: both of the NT constructs express reasonably well, and both can interact with mGluR1 such that they are retained on the cell surface.

Recent reports have suggested that mGluR activation involves a conformational change in which each dimer subunit pivots with respect to the other on ligand binding and VFT “closing” (Jingami et al. 2003; Sato et al. 2003). This change may require plasma membrane anchoring of both subunits to occur and to translate into G protein activation (Kunishima et al. 2000; Rondard et al. 2006). The data presented here, in which an unanchored mGluR1 VFT acts as a dominant negative subunit, support this model (Rondard et al. 2006; Sato et al. 2003).

In addition to the effects of myc-mG1-NT on group I mGluRs, the effect on mGluR1 C140G was tested. Mutation of the C140 residue of mGluR1 and of the corresponding cysteine in mGluR5 results in a point mutant that lacks covalent dimerization (Ray and Hauschild 2000) but likely retains the ability to form noncovalent dimers (Romano et al. 2001) and appears to function normally. It remains unclear, however, whether mGluR1 C140 mutants signal as dimers, as monomers, or both. Further, it is not known how stable mGluR1 C140 mutant dimers are, or whether they occupy some equilibrium between monomeric and dimeric states in live cells. Because the myc-mG1-NT construct could occlude mGluR1 signaling presumably by disrupting dimerization, the opportunity to address these questions was presented. Indeed myc-mG1-NT was as potent a dominant negative against mGluR1 C140G as against mGluR1, indicating that the C140 mutant does indeed derive its ability to signal from a dimeric state. Otherwise, it’s likely that signaling would be strong, or at least moderate, in the presence of the myc-mG1-NT subunit.

Interestingly, although myc-mG1-NT acted to potently occlude mGluR1 C140G activity, retention of myc-mG1-NT on the plasma membrane was not detectable when co-expressed with mGluR1 C140G. These data suggested that while the VFT of mGluR1 may prevent signaling of mGluR1 and mGluR1 C140G by an analogous mechanism, the basic interaction between these subunits is different. These data are consistent with the interpretation that like the wild-type mGluR1 (Robbins et al. 1999), mGluR1 C140G dimerizes prior to plasma membrane insertion (e.g., in the ER or Golgi). Thus preventing this dimerization causes loss of function. Unlike the myc-mG1-NT/mGluR1 interaction, however, the myc-mG1-NT/mGluR1 C140G interaction appears reversible, hence the lack of detectable myc-mG1-NT retention. It may be inferred then that reformation of mGluR1 C140G dimers on the plasma membrane does not occur or occurs very slowly such that no signaling is detectable when myc-mG1-NT is co-expressed. Thus these data argue against a model in which mGluR1 C140 mutants exhibit dynamic equilibrium between monomeric and dimeric states in living cells and therefore probably exist, or at least signal, primarily as dimers.

The final set of experiments provide further support for these conclusions by demonstrating that the DI-pep, which consists of a 20 amino acid alpha helix that makes up a considerable portion of the dimer interface (Jingami et al. 2003; Sato et al. 2003) fails to disrupt mGluR1 C140G signaling (and therefore dimerization) when applied acutely to the bath, even for prolonged periods of $\geq 2$ h. This peptide can, however, disrupt mGluR1 C140G signaling (and thus presumably dimerization) when injected into cells several hours before the experiments but failed to disrupt wild-type mGluR1 under any conditions tested. While the inability of the DI-pep to disrupt wild-type mGluR1 dimerization (signaling) was unsurprising due to the covalent bond linking mGluR1 dimers, the inability to disrupt mGluR1 C140G signaling provides further support for the interpretation that this mutant primarily occupies and signals from the dimeric state when expressed in live cells rather than in an equilibrium between monomers and dimers. Otherwise, the peptide would be expected to impart at least some inhibition of signaling when applied extracellularly at the relatively high concentration of 10 $\mu$M, the same concentration that was shown to disrupt the mutant receptor when injected intracellularly.

It should be noted that although the mechanism of action of the DI-pep is assumed to be through prevention of dimerization by occupying the dimer interface surface on a full-length receptor, this assumption cannot be directly verified. The lack of effect on wild-type mGluR1 provides a control against the interpretation that the peptide works through some nonspecific mechanism, such as inhibition of expression. But while it is assumed that the differential effects observed with mGluR1 and mGluR1 C140G arise from the loss of a covalent interaction to link the dimers in the mutant, it is difficult to predict the impact on the mGluR1 structure caused by the mutation because the published structure (Jingami et al. 2003) leaves a stretch of $\sim 20$ nearby amino acids unresolved, including the C140 residue, which one assumes must come into close proximity with the opposing dimer subunit at least for the wild-type receptor. Thus a large change in the structure in
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Neyman S, Manahan-Vaughan D. Metabotropic glutamate receptor 1 (mGlur1) and 5 (mGlur5) regulate late phases of LTP and LTD in the hippocampal CA1 region in vivo. Eur J Neurosci 27: 1345–1352, 2008.


Tsuchiya D, Kunishima N, Kamiya N, Jingami H, Morikawa K. Structural views of the ligand-binding cores of a metabotropic glutamate receptor.

N this area may underlie the differential effect of the receptor in addition to or perhaps instead of the loss of the covalent disulfide bond.

Finally, perhaps the most interesting implication of the data presented here is the finding that a truncated mGlur consisting mainly of the VFT can act as a dominant negative subunit. Indeed splice variants of several mGlus including mGlur1, mGlur6, and mGlur8 have been identified that consist of only the extracellular domains of these receptors (Malherbe et al. 1999; Valerio et al. 2001; Zhu et al. 1999). These variants have been proposed to act extracellularly to perhaps buffer glutamate in the synaptic cleft. However, if these endogenous proteins act like the myc-mG1-NT construct examined here, expression of these variants may provide a means for cells to silence signaling through specific mGlur subtypes, a potentially more physiologically important function. Our data suggest that the VFT splice variant of mGlur1 silences both mGlur1 and mGlur5 and has no effect on mGlur2, but its effect on other mGlus is not known. Likewise, the effects of the mGlur6 and mGlur8 VFT splice variants on signaling of various mGlus is unknown, but it is interesting to speculate that each may have the ability to silence a single or small subset of the mGlus expressed natively in a particular tissue.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


