

4-Methylhomoibotenic Acid Activates a Novel Metabotropic Glutamate Receptor Coupled to Phosphoinositide Hydrolysis¹

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ABSTRACT

Metabotropic glutamate receptors (mGluRs) are a family of glutamate receptors that are coupled to a variety of second messenger systems through GTP-binding proteins. Of the eight subtypes cloned to date, mGluR1 and mGluR5 are coupled to phosphoinositide hydrolysis in expression systems, and both are activated by the glutamate analogue 1-aminocyclopentane-1S,3R-dicarboxylic acid. Previously, we provided evidence that in rat cortical slices, 4-bromohomoibotenic acid (BrHI) and 4-methylhomoibotenic acid (MHI) activate a 1-aminocyclopentane-1S,3R-dicarboxylic acid-insensitive phosphoinositide hydrolysis-coupled mGluR. We further examine these compounds in expression systems. In a stable cell line expressing mGluR1a, BrHI is a weak partial agonist whereas MHI has no agonist activity. In *Xenopus* oocytes expressing mGluR1a or

mGluR5a, BrHI is a weak agonist at mGluR5a whereas MHI is without effect on either receptor. Both BrHI and MHI have weak agonist activity at mGluRs 4a and 7a expressed in stable BHK cell lines whereas neither compound had any activity on BHK cells expressing mGluR2. Finally, we found that the novel mGluR antagonist LY341495 completely blocked the activation of mGluR1 and mGluR5 and blocked the phosphoinositide hydrolysis response to DHPG in rat cortical slices. In contrast, LY341495 did not block the phosphoinositide hydrolysis response to MHI in rat cortical slices. This provides further evidence that the phosphoinositide hydrolysis response to MHI in rat cortical slices is due to activation of a novel receptor that is distinct from the previously cloned mGluRs.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and can transduce its effects through two major classes of receptors, the iGluRs and the mGluRs. The mGluRs are coupled through heterotrimeric G-proteins to a variety of signal transduction systems (Conn *et al.*, 1995) and have been shown to play a role in modulating neuronal excitability and synaptic transmission in a number of regions within the mammalian central nervous system.

To date, genes encoding eight different mGluR subtypes have been cloned (designated mGluR1 through mGluR8) (see Conn and Pin, 1997; Pin and Duvoisin, 1995 for reviews). The mGluRs have been divided into three major groups based on

sequence homology, pharmacology and second messenger coupling in expression systems. Group I mGluRs consist of mGluR1, mGluR5 and their splice variants, and couple to the activation of phospholipase C and the hydrolysis of membrane phosphoinositides in a variety of expression systems. Group II mGluRs include mGluR2 and mGluR3, which negatively couple to adenylyl cyclase in expression systems. Group III consists of mGluR4, mGluR6, mGluR7 and mGluR8 which also negatively couple to adenylyl cyclase in expression systems (Conn and Pin, 1997). Members of a group show more than 60% homology with other members of the same group and approximately 40% homology with members of different groups.

Increasing evidence suggests that novel mGluRs may exist in brain that do not correspond to mGluRs 1–8. For instance, we recently reported that two glutamate analogs BrHI and MHI activate phosphoinositide hydrolysis in rat cortical

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ABBREVIATIONS: 1S,3R-ACPD, 1-aminocyclopentane-1S,3R-dicarboxylic acid; ABHD, (1RS, 2SR, 4RS, 7RS)-amino-bicyclo [2.2.1] heptane dicarboxylate; AIDA, 1-aminoindan-1,5-dicarboxylate; BHK, baby hamster kidney; BrHI, 4-bromohomoibotenic acid; CPPG, (RS)- α -cyclopropyl-4-phosphonophenylglycine; DHPG, dihydroxyphenylglycine; DMEM, Dulbecco's minimal essential medium; E-Glu, (2S)- α -ethylglutamic acid; iGluR, ionotropic glutamate receptor; KRB, Krebs bicarbonate buffer; L-AP4, L-2-amino-4-phosphonobutyric acid; MAP4, α -methyl-L-2-amino-4-phosphonobutyric acid; MCCG, (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine; MCPG, (R,S)- α -methyl-4-carboxyphenylglycine; mGluR, metabotropic glutamate receptor; MHI, 4-methylhomoibotenic acid; MPPG, (R,S)- α -methyl-4-phosphonophenylglycine; MSOP, (R,S)- α -methylserine-O-phosphate; MSOPPE, (R,S)- α -methylserine-O-phosphate momophenyl ester; MSPG, (R,S)- α -methyl-4-sulphonophenylglycine; MTPG, (R,S)- α -methyl-4-tetrazolylphenylglycine; PLD, phospholipase D; R,S-4C3HPG, (R,S)-4-carboxy-3-hydroxyphenylglycine; (S)-4C3HPG, (S)-4-carboxy-3-hydroxyphenylglycine.

slices in the presence of ionotropic glutamate receptor antagonists (Chung *et al.*, 1994). This phosphoinositide hydrolysis response is completely additive with the phosphoinositide hydrolysis response of 1S,3R-ACPD, a nonselective mGluR agonist active at mGluR1 and mGluR5 as well as other mGluRs. Furthermore, BrHI did not elicit other responses known to be mediated by mGluRs in rat brain slices, including inhibition of forskolin-stimulated cAMP accumulation, potentiation of vasoactive intestinal peptide-induced cAMP responses, or activation of PLD, suggesting that these compounds may be selective for the ACPD-insensitive phosphoinositide hydrolysis-linked receptor relative to other mGluRs. However, the effects of BrHI and MHI on the cloned mGluRs have not been directly determined. Here, we further characterize these glutamate analogs by testing their effects on cloned mGluRs in expression systems. We also compare the effects of various antagonists on the phosphoinositide hydrolysis response to submaximal concentrations of the selective group I mGluR agonist, DHPG and MHI. These studies provide further support for the hypothesis that BrHI and MHI activate a novel mGluR subtype that is distinct from any of the previously cloned mGluRs. Furthermore, these studies suggest that MHI is more selective than BrHI as an agonist of the novel mGluR.

Materials and Methods

Measurement of phosphoinositide hydrolysis. Phosphoinositide hydrolysis was measured in BHK cells stably expressing mGluR1a using a modified version of the method described by Conn and Wilson (1991). In brief, cells were grown in DMEM supplemented with penicillin, streptomycin, glutamine and heat-inactivated fetal bovine serum and plated into 12-well plates. Cells were incubated in a constant temperature (37°C), constant atmosphere (5% CO₂) incubator. 24 to 48 hr before an experiment, cells were incubated in 1 μ Ci/well of [³H]inositol. On the day of the experiment, cells were washed at least three times using KRB (108 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM glucose and 25 mM NaHCO₃) supplemented with 10 mM LiCl. Cells were allowed to incubate in the LiCl supplemented KRB for 15 min, after which agonists were added and cells were again allowed to incubate for an additional 45 min. The reaction was stopped by adding 600 μ l of methanol and each well was scraped using a plastic policeman into a test tube. A total of 600 μ l of chloroform and 300 μ l 0.5 M HCl was added to separate the aqueous and organic phases with vortex-mixing for 1 min and low-speed centrifugation. An aliquot of the aqueous phase (750 μ l) was added to anion exchange columns containing Dowex-1 (200–300 mesh in the formate form) for separation of [³H]inositol-containing compounds. [³H]inositol monophosphate was eluted directly into scintillation vials, and the radioactivity present was determined by liquid scintillation counting using Fisher's BioHP scintillant. A similar protocol was used for measuring phosphoinositide hydrolysis in hippocampal slices as previously described (Chung *et al.*, 1994).

Measurement of cyclic AMP accumulation. cAMP accumulation was measured in stable BHK cell lines expressing mGluRs using a modification of the method of Shimizu *et al.* (1969) as previously described (Johnson and Minneman, 1987; Winder and Conn, 1992) with additional modifications. The conversion of [³H]adenine to [³H]cAMP was measured. Cells were plated into 12-well plates (each well = 22.1 mm in diameter) 3 to 4 days before the assay. On the day of the assay, each well was incubated in 1 ml of oxygenated (95% O₂, 5% CO₂) Krebs' bicarbonate buffer (108 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM glucose and 25 mM NaHCO₃) containing 1 μ Ci of [³H]adenine for 2 hours. After

incubation, cells were washed three times with 1 ml fresh warmed KRB. A total of 200 μ M IBMX and 10 μ M forskolin in addition to the appropriate agonists/antagonists was added to each well to achieve a final volume of 500 μ l. Cells were then incubated for 15 min at 37°C.

The reaction was terminated with 50 μ l 77% trichloroacetic acid and 25 μ l of 10 mM cAMP as a carrier. The fluid in each tube was then transferred into centrifugation tubes. Each well was washed with an additional 250 μ l KRB, which was also transferred to the centrifugation tubes. The tubes were then centrifuged at 30,000 \times g for 15 min.

The radioactivity present in 25- μ l aliquots of the supernatant from each tube was determined using liquid scintillation counting and was used to determine the radioactivity incorporated into the cells. The remaining supernatant of each tube was poured over Dowex columns (Sigma Chemical Co., St. Louis, MO). The columns were then washed with 3 ml of deionized water and then placed over alumina columns. Next, 5 ml of deionized water were added. Finally [³H]cAMP was eluted from the alumina columns into scintillation vials with 2 ml of 50 mM Tris buffer (pH 8.0). The radioactivity present (which represents the conversion of [³H]adenine to [³H]cAMP) was determined by liquid scintillation counting.

Preparation and injection of oocytes. DNA template for mGluR5a were made from bluescript SK-vectors containing the appropriate clone generously supplied by Dr. S. Nakanishi (Kyoto University). mGluR1a template was made from the clone originally supplied by Dr. Nakanishi in bluescript SK+ ligated into pCDM8. DNA template were purified using the alkaline-lysis protocol (Sambrook *et al.*, 1989). Finally, a T7 synthesis kit was used to synthesize RNA from the templates. Rat cortex mRNA was prepared according to the instructions included in the Fasttrack kit from Invitrogen (Carlsbad, CA).

Xenopus oocytes (stages V and VI of development), 24 hr after treatment with collagenase, were microinjected with 0.1 ng of mGluR1a RNA, 10 ng of mGluR5a RNA or 75 ng of whole cortex RNA in a volume of 50, 50 and 75 nl, respectively. The oocytes were stored in a 17°C incubator in Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 15 mM HEPES, 0.32 mM CaNO₃, 0.4 mM CaCl₂, 0.81 mM MgSO₄ plus penicillin and streptomycin). Electrical recordings were made 3 to 7 days after injection using a two-electrode voltage clamp and the oocyte clamp amplifier by Warner Instruments (Hamden, CT). The recording chamber was continuously perfused with oocyte recording solution (90 mM NaCl, 1 mM KCl, 10 mM HEPES, 1.5 mM CaCl₂, 1.5 mM MgCl₂). All recordings were from a holding potential of -50 or -60 mV. Drugs were prepared in separate bottles and bath applied. Flow of solutions was approximately 1 ml/min. Data were digitized and analyzed off line.

Materials. L-AP4, MCPG, AIDA, (S)-4C3HPG, R,S-4C3HPG, MCCG, MTPG, MSPG, MPPG, MSOPPE, MSOP, MAP4, CPPG, E-Glu and ABHD were purchased from Tocris Cookson (St. Louis, MO). LY341495 was graciously provided by Dr. Paul Ornstein (Eli Lilly; Indianapolis, IN). Atropine, prazosin, ketanserin and mepyramine were purchased from Sigma (St. Louis, MO). Stable cell lines expressing mGluR1a and 4 were generously supplied by Betty Haldeman (Xymogenetics, Seattle, WA); cell lines expressing mGluR2 by Dr. Shigetada Nakanishi (Kyoto University; Kyoto, Japan); and cells expressing mGluR7 by Dr. Tom Segerson (Vollum Institute; Portland, OR).

Results

The effect of BrHI and MHI on group I receptors in expression systems. Consistent with previous reports, glutamate elicited a robust, concentration-dependent increase in the accumulation of inositol monophosphate in BHK cells stably transfected with mGluR1a. The maximal increase in phosphoinositide hydrolysis elicited by 1 mM glutamate represented a response that was 29.0 \pm 2.8 (mean \pm S.E.M.)

times the basal response in the absence of added agonist. BrHI also elicited a small, but significant concentration-dependent increase in the accumulation of inositol mono-phosphate ($EC_{50} = 165 \mu\text{M}$; fig. 1). The maximal response elicited by BrHI was approximately $25\% \pm 4.67$ (all values are mean \pm S.E.M.) of the maximal response to glutamate. In contrast to BrHI, MHI, at concentrations up to 1 mM, had no significant effect on phosphoinositide hydrolysis in cells expressing mGluR1a. Therefore, BrHI behaves as a partial agonist at mGluR1a whereas MHI has no agonist activity at this receptor.

To further determine the effects of these compounds on mGluR1a, cRNA (0.1 ng/50 nl H_2O) for mGluR1a was injected into *Xenopus* oocytes and the amplitude of the calcium-dependent chloride current elicited by these compounds was measured (fig. 2). We chose to study the effect of $316 \mu\text{M}$ BrHI and 1 mM MHI because these concentrations are maximally effective at stimulating phosphoinositide hydrolysis in rat cortical tissue (Chung *et al.*, 1994). In contrast to its effect on BHK cells expressing mGluR1a, BrHI did not elicit a discernable mGluR1a-mediated response in *Xenopus* oocytes. MHI was also without effect in this expression system. The mean current elicited by BrHI and MHI in oocytes injected with mGluR1 RNA was $-15.2 \text{ nA} \pm 11.9$ ($n = 8$) and $9.3 \text{ nA} \pm 1.9$ ($n = 4$) respectively, compared to a mean current of $-1881.2 \text{ nA} \pm 465.9$ ($n = 8$) induced by $100 \mu\text{M}$ L-glutamate.

To complete the examination of group I mGluRs, we injected mGluR5a cRNA (10 ng/50 nl) into *Xenopus* oocytes as well. BrHI is a clear agonist at mGluR5a, whereas MHI again elicited no detectable response with a mean current of $-0.85 \text{ nA} \pm 7.824$ ($n = 3$) (fig. 2). In oocytes injected with mGluR5a cRNA, $316 \mu\text{M}$ BrHI elicited a mean current of $-210.4 \text{ nA} \pm 69.1$ ($n = 10$) compared to a mean current of $-645.0 \text{ nA} \pm 151.9$ ($n = 10$) induced by $100 \mu\text{M}$ L-glutamate. These data suggest that BrHI is an agonist at both mGluR1a and mGluR5 whereas MHI is inactive at both of these receptors (fig. 2).

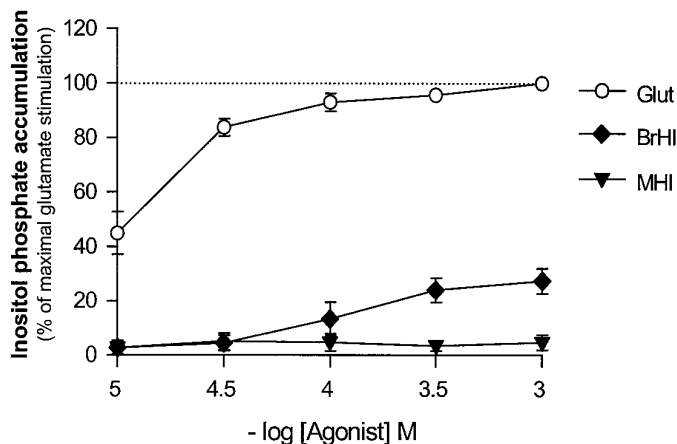


Fig. 1. Effect of increasing concentrations of glutamate, BrHI and MHI on [^3H]inositol phosphate accumulation in a stable BHK cell line expressing mGluR1a. Cells were incubated with the appropriate agonist for 45 min in the presence of LiCl. Results are presented as a percentage of maximal stimulation of phosphoinositide hydrolysis by 1 mM glutamate, which was 29.0 ± 2.8 times the basal response (radioactivity in [^3H]inositol phosphate) in the absence of added agonist. Data are mean \pm S.E.M. (bars) values from three separate experiments, each performed in triplicate.

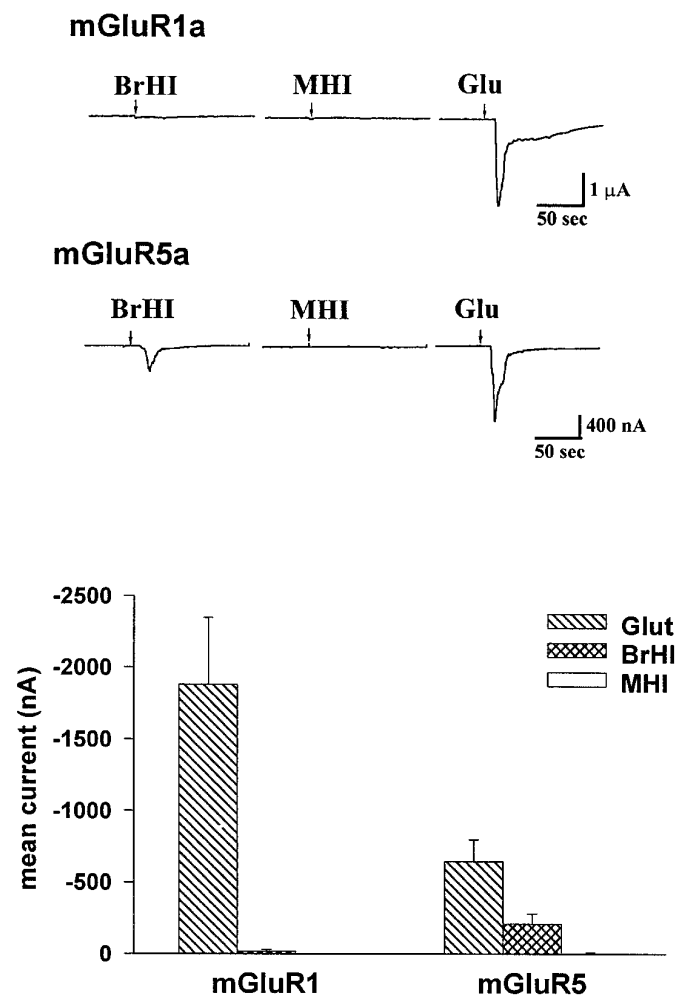


Fig. 2. Effect of glutamate ($100 \mu\text{M}$), BrHI ($316 \mu\text{M}$) and MHI (1 mM) on *Xenopus* oocytes injected with mGluR1a or mGluR5a cRNA. On top are representative traces from oocytes injected with mGluR1a or mGluR5a cRNA, respectively, challenged with each of the three agonists. Below results are presented as mean \pm S.E.M. (bars) from multiple experiments. For glutamate or BrHI on mGluR1, $n = 8$; MHI on mGluR1, $n = 4$; glutamate or BrHI on mGluR5, $n = 10$; and MHI on mGluR5, $n = 3$.

The effect of BrHI and MHI on mGluR2 stably expressed in BHK cells. We next determined the effect of BrHI and MHI on mGluR2, a group II mGluR. In a stable BHK cell line expressing mGluR2, forskolin elicited an accumulation of cAMP that was 2.6 ± 0.38 times the basal accumulation of cAMP. Glutamate elicited a $75.8\% \pm 13.8$ reduction in the forskolin ($10 \mu\text{M}$) induced increase in cAMP accumulation (fig. 3). In contrast, high concentrations (1 mM) of either MHI or BrHI had no significant effect on forskolin-stimulated increase in cAMP accumulation (fig. 3), suggesting that neither compound is an mGluR2 agonist. This is consistent with a previous report that BrHI is inactive at mGluR2 (Thomsen *et al.*, 1994a).

The effect of BrHI and MHI on group III receptors stably expressed in BHK cells. We next tested the effects of BrHI and MHI on group III mGluRs. In BHK cells stably transfected with mGluR4a, the mean cAMP response to forskolin was 6.8 ± 0.04 times basal cAMP accumulation. In these cells, 1 mM L-AP4 induced an $87.9\% \pm 9.8$ inhibition of the forskolin-stimulated cAMP accumulation. In addition, both BrHI and MHI were effective at inhibition of the fors-

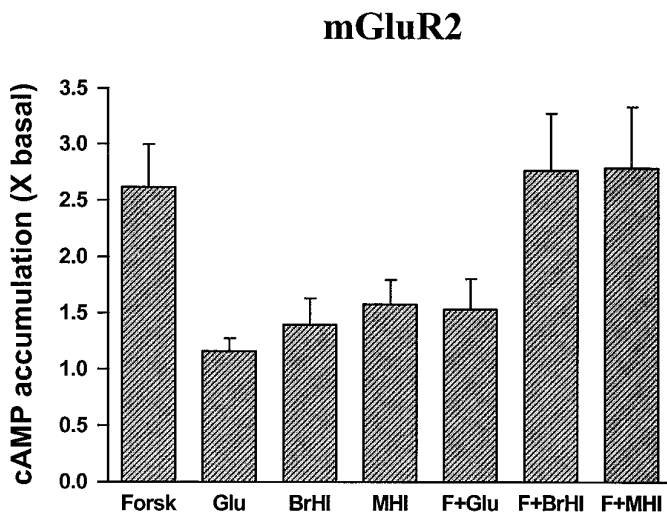


Fig. 3. Effect of glutamate (1 mM), BrHI (1 mM) and MHI (1 mM) on 10 μ M forskolin-stimulated cAMP accumulation in a stable BHK cell line expressing mGluR2. Cells were incubated with the appropriate agonists for 15 min. Results are expressed as multiples of basal cAMP accumulation. Data are mean \pm S.E.M. (bars) values from at least three separate experiments, each performed in triplicate.

kolin stimulated response. One mM BrHI induced a $51.4\% \pm 17.6$ inhibition of the forskolin-stimulated cAMP accumulation whereas MHI induced a $35.0\% \pm 12.4$ inhibition (fig. 4).

In BHK cells stably transfected with mGluR7a, the mean forskolin response was 4.04 ± 0.29 times the basal accumulation of cAMP. In these cells, 1 mM L-AP4 inhibited forskolin stimulated cAMP accumulation by $70.0\% \pm 5.9$. Further, 1 mM BrHI induced a $28.0\% \pm 6.4$ inhibition although 1 mM MHI was more effective and elicited a $53.6\% \pm 9.1$ inhibition of the forskolin-stimulated response. These data suggest that both BrHI and MHI can activate group III mGluRs.

Lack of synergy between MHI and ACPD on mGluR1 or mGluR5. The previous finding that MHI elicits a phosphoinositide hydrolysis response in rat cortical slices that is additive with the response to 1S,3R-ACPD (Chung *et al.*, 1994), coupled with our finding that MHI does not activate group I mGluRs suggests that MHI is capable of activating a novel ACPD-insensitive mGluR that is coupled to phosphoinositide hydrolysis. However, it is also possible that MHI could in some way interact with group I mGluRs and potentiate the response to glutamate or ACPD. Thus, we examined the effect of MHI on the activation of mGluR1a or mGluR5a by ACPD in *Xenopus* oocytes.

In either mGluR1a or mGluR5a cRNA-injected oocytes, we found that 1 mM MHI, in the presence of 100 μ M CNQX, did not potentiate responses to 1 mM 1S,3R-ACPD (fig. 5). The response to coapplication of both agonists was in no case larger than application of 1 mM 1S,3R-ACPD alone. A concentration of 100 μ M glutamate, in the same oocytes, elicited a current that was larger in amplitude than that elicited by 1 mM 1S,3R-ACPD, indicating that the calcium-activated Cl⁻ current was not saturated by 1 mM 1S,3R-ACPD alone (data not shown). Therefore, it is likely that the phosphoinositide hydrolysis response elicited in rat cortical slices by MHI is not due to activation of mGluR1a or mGluR5a, or a potentiation of the response of these receptors to ACPD. Finally, although it appears that the mean response to MHI and 1S,3R-ACPD added together on mGluR1a expressing oocytes

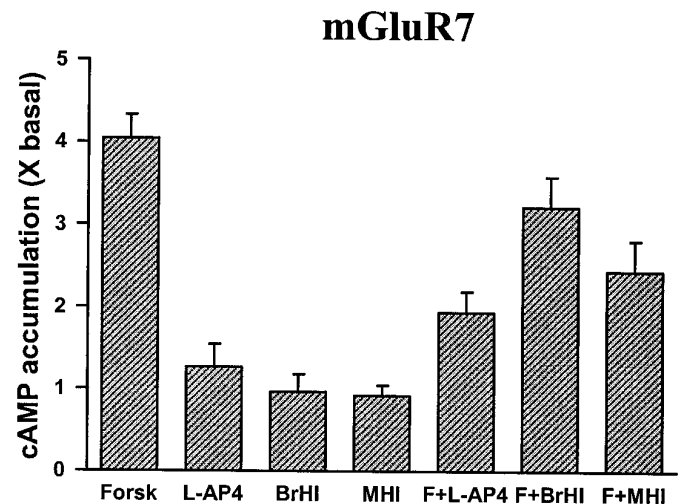
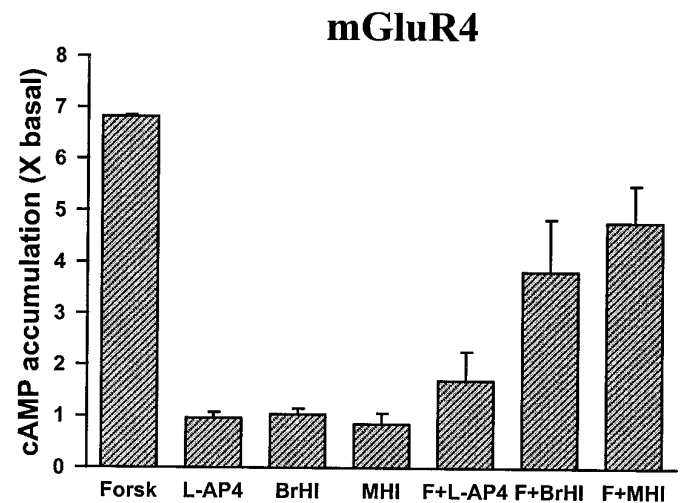


Fig. 4. Effect of L-AP4 (1 mM), BrHI (1 mM) and MHI (1 mM) on 10 μ M forskolin-stimulated cAMP accumulation in a stable BHK cell lines expressing mGluR4 and mGluR7. Cells were incubated with the appropriate agonists for 15 min. Results are expressed as multiples of basal cAMP accumulation. Data are mean \pm S.E.M. (bars) values from at least three separate experiments, each performed in triplicate.

was less than the current elicited by 1S,3R-ACPD alone, the two groups were not statistically different from one another ($P = .11$, *t* test).

The effect of mGluR antagonists on the phosphoinositide hydrolysis response to DHPG and MHI. The finding that MHI has no agonist effect on mGluR1 and mGluR5 is consistent with the hypothesis that this compound activates a novel receptor that is distinct from the previously cloned mGluRs. If this is the case, it is possible that the MHI-sensitive receptor and group I mGluRs may be differentially sensitive to putative mGluR antagonists. Thus, we determined the effects of various putative mGluR antagonists on the phosphoinositide hydrolysis response to MHI and the group I-selective agonist DHPG in rat cortical slices. Using EC_{75} concentrations of MHI and DHPG (600 and 80 μ M, respectively), we tested 1 mM of a variety of known mGluR antagonists. None of the antagonists gave a significant blockade of the response to MHI (fig. 6). However, MCPG, a known antagonist of group I mGluRs, gave an

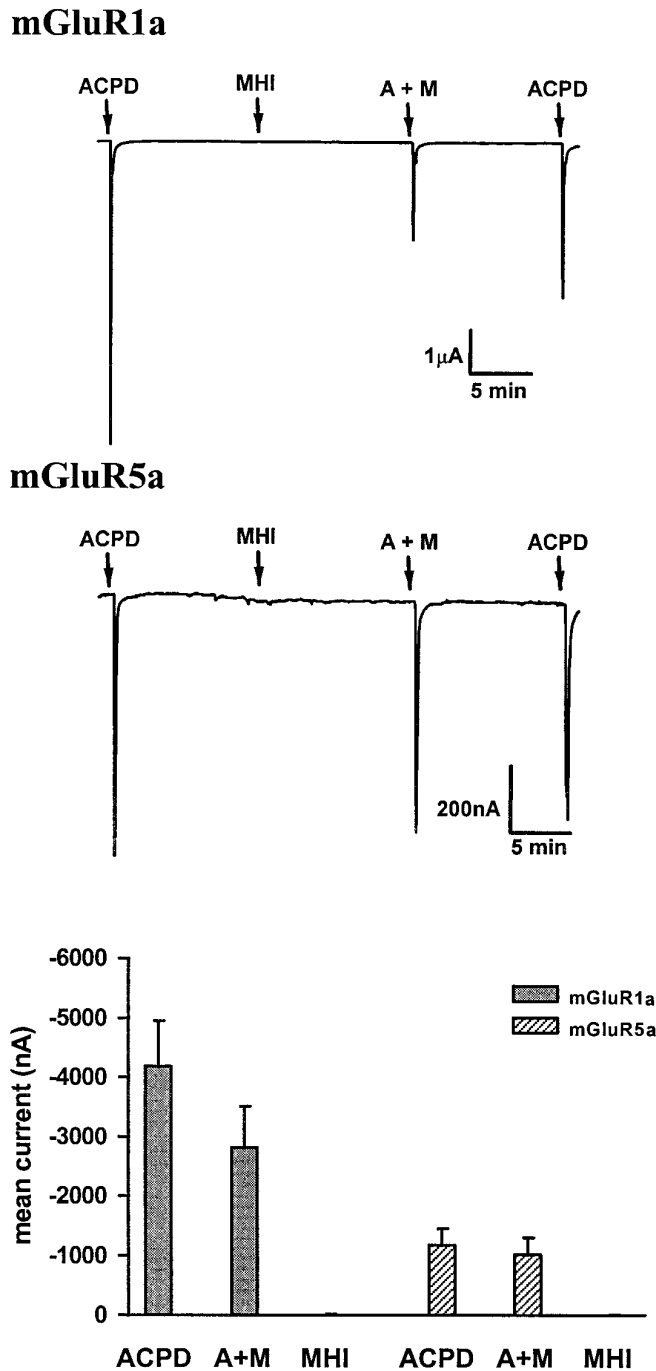


Fig. 5. Lack of potentiation of 1S,3R-ACPD (1 mM) induced Cl⁻ current in *Xenopus* oocytes injected with mGluR1a cRNA or mGluR5a cRNA by 1 mM MHI. At left are representative traces from oocytes injected with mGluR1a or mGluR5a cRNA. Agonists or combination of agonists were applied 12 min apart in a randomized order. At right, results are means ± S.E.M. (bars) from six separate experiments.

approximately 50% blockade of the phosphoinositide hydrolysis response to DHPG ($P = .05$, one-tailed t test). Furthermore, a new mGluR antagonist, LY341495 (Ornstein *et al.*, 1996), completely blocked the phosphoinositide hydrolysis response to DHPG ($P < .01$, one-tailed t test) although the same concentration (1 mM) had no effect on the phosphoinositide hydrolysis response elicited by MHI ($P = .47$, one-tailed t test) (fig. 6).

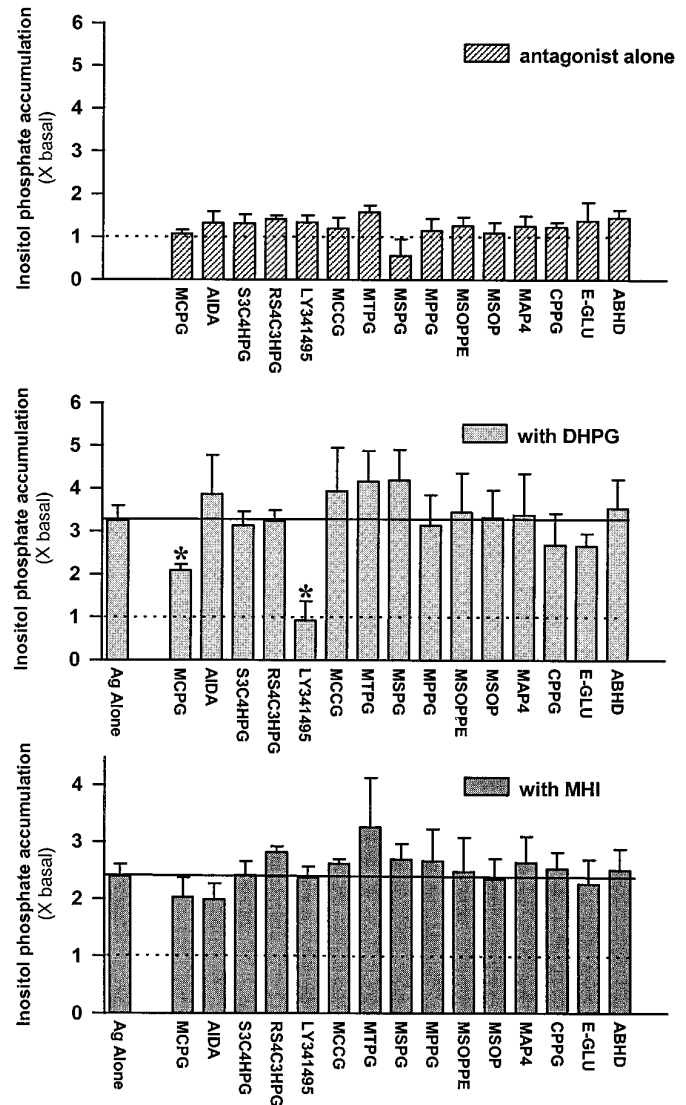


Fig. 6. Effect of 1 mM of a variety of mGluR antagonists on the phosphoinositide hydrolysis response to EC₇₅ concentrations of DHPG (80 μ M) or MHI (600 μ M) in rat cortical slices in the presence of 100 μ M CNQX and 100 μ M D-AP5. Tissue was incubated with the appropriate drug(s) for 45 min in the presence of LiCl and iGluR antagonists. Results are presented as radioactivity in [³H]inositol phosphate from slices incubated with a given concentration of drug divided by radioactivity in [³H]inositol phosphate from slices incubated in the absence of added drugs (basal radioactivity). Data are mean ± S.E.M. (bars) values from three separate experiments, each performed in triplicate. Asterisks represent a significant difference in the mean from agonist alone ($P < .05$, 1 tailed t test).

Concentration response studies in rat cortical slices revealed that LY341495 induces a dose-dependent blockade of the phosphoinositide hydrolysis response to 80 μ M DHPG (fig. 7), completely blocking the response with an IC₅₀ of 36.9 μ M. In addition, we found that LY341495 could also completely block the phosphoinositide hydrolysis response to ACPD in slices (data not shown). Because the effects of LY341495 has not yet been characterized on the group I mGluRs, we next wanted to determine directly the effect of LY341495 on mGluR1a and mGluR5a. We found that LY341495 induced a concentration-dependent blockade of the phosphoinositide hydrolysis response to an EC₇₅ concen-

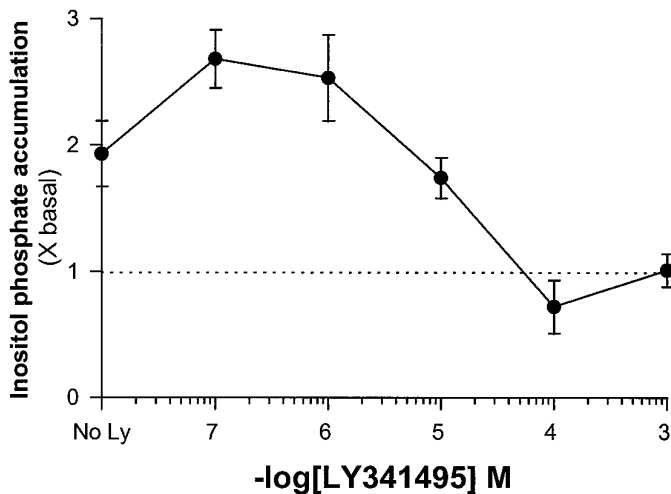


Fig. 7. Dose response of LY341495 on the phosphoinositide hydrolysis response to an EC_{75} concentration of DHPG ($80 \mu\text{M}$) in rat cortical slices. Tissue was incubated with the appropriate drug(s) for 45 min in the presence of LiCl and iGluR antagonists. Results are presented as radioactivity in [^3H]inositol phosphate from slices incubated with a given concentration of drug divided by radioactivity in [^3H]inositol phosphate from slices incubated in the absence of added drugs (basal radioactivity). Data are mean \pm S.E.M. (bars) values from three separate experiments, each performed in triplicate.

tration of glutamate ($40 \mu\text{M}$) on stable cell lines expressing mGluR1a (fig. 8). Furthermore, $100 \mu\text{M}$ LY341495 reversibly blocked the calcium-dependent chloride current elicited by $50 \mu\text{M}$ glutamate in *Xenopus* oocytes with mGluR5a cRNA. Taken together, these data suggest that LY341495 is an antagonist of group I mGluRs in native tissue and in expression systems. Thus, the finding that MCPG and LY341495 do not block the phosphoinositide hydrolysis response to MHI in cortical slices provides further evidence that the response to MHI is not mediated by activation of one of the known phosphoinositide hydrolysis-linked mGluRs.

In addition to the mGluR antagonists, we also found that 1 mM prazosin, ketanserin, atropine or mepyramine were not able to block the phosphoinositide hydrolysis response to $600 \mu\text{M}$ MHI (data not shown).

Discussion

Although eight mGluR subtypes have been identified by molecular cloning, there is mounting evidence that more mGluR subtypes exist in rat brain that have not yet been cloned. For instance, 1S,3R-ACPD and other mGluR agonists activate a phospholipase D-coupled receptor in rat hippocampal slices (Boss and Conn, 1992; Boss *et al.*, 1994; Holler *et al.*, 1993). Although the pharmacological profile of this receptor is similar to that of other mGluRs, it is clearly distinct from that of any of the previously cloned mGluR subtypes (Boss *et al.*, 1994; Pellegrini-Giampietro *et al.*, 1996). In addition, Scholz (1994) recently described a phosphoinositide hydrolysis-linked mGluR in cultured hippocampal pyramidal neurons that is activated by glutamate and ibotenate but is insensitive to 1S,3R-ACPD. In cortical neuronal cultures, Thomsen *et al.* (1993) found that ACPD was unable to stimulate phosphoinositide hydrolysis although glutamate could elicit a 6-fold increase. Because mGluR1 and mGluR5 are both fully activated by 1S,3R-ACPD, these may represent

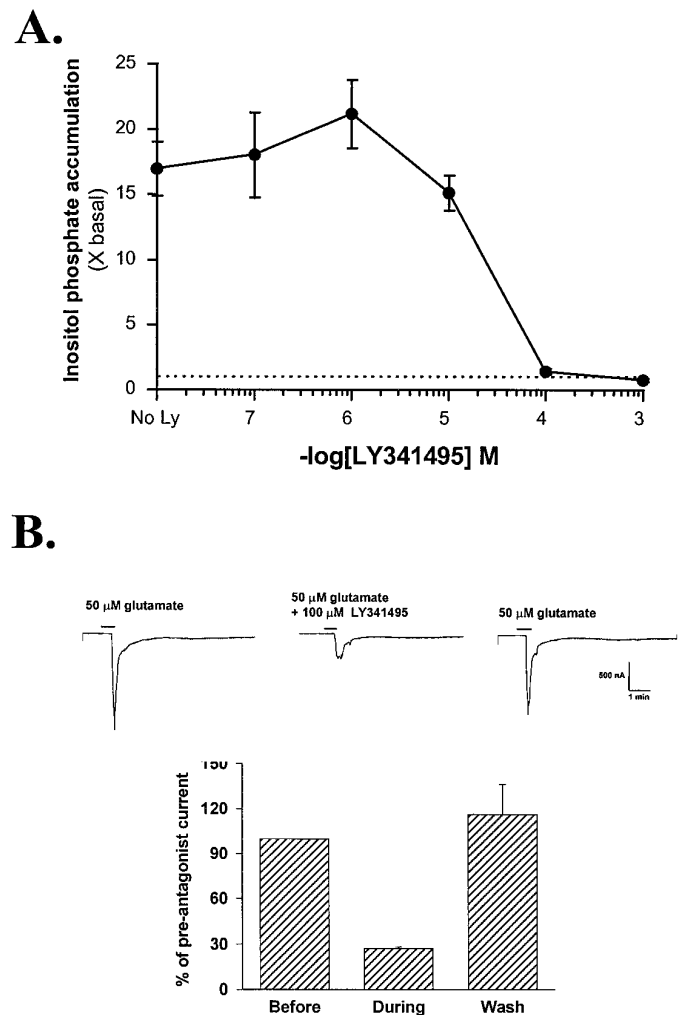


Fig. 8. A, Dose response of LY341495 on the phosphoinositide hydrolysis response to the EC_{75} concentration of glutamate ($40 \mu\text{M}$) in BHK cells expressing mGluR1a. Cells were incubated with the appropriate drug(s) for 45 min in the presence of LiCl. Results are presented as radioactivity in [^3H]inositol phosphate from slices incubated with a given concentration of drug divided by radioactivity in [^3H]inositol phosphate from cells incubated in the absence of added drugs (basal radioactivity). Data are mean \pm S.E.M. (bars) values from three separate experiments, each performed in triplicate. B, Effect of LY341495 on *Xenopus* oocytes injected with mGluR5a cRNA. $100 \mu\text{M}$ LY341495 reversibly inhibited the current elicited by a submaximal concentration of glutamate ($50 \mu\text{M}$). Applications were performed in the same oocyte, with drug applications 30 min apart. The above trace is representative of at least three separate experiments performed in the same manner. Mean (\pm S.E.M.) data across experiments are shown below.

responses to activation of a novel phosphoinositide hydrolysis-linked mGluR. Finally, electrophysiological responses to mGluR agonists have been described in cortex (Mannaioni *et al.*, 1996) and dorsolateral septal nucleus (Zheng *et al.*, 1995; Zheng and Gallagher, 1995) that have pharmacological profiles that are distinct from those of any cloned mGluRs.

In our studies we provide further evidence for the existence of a novel phosphoinositide hydrolysis-linked mGluR that is activated by two glutamate analogs, BrHI and MHI. Evidence in support of the existence of this receptor includes the previous finding that BrHI and MHI activate phosphoinositide hydrolysis in rat cortical slices and that the response to BrHI and MHI is completely additive with that of 1S,3R-

ACPD (Chung *et al.*, 1994) or the group I-selective agonist DHPG (D. S. Chung and P. J. Conn, unpublished data). We now report that MHI has no agonist effect on either mGluR1a or mGluR5a, although BrHI can at least partially activate both mGluR1a and mGluR5a. These findings are consistent with the previous finding that BrHI inhibits glutamate-stimulated phosphoinositide hydrolysis in BHK cells expressing mGluR1a (Thomsen *et al.*, 1994a), because we found that BrHI is only a partial agonist at this receptor.

The finding that MHI has no agonist activity at mGluR1 or mGluR5 suggests that activation of phosphoinositide hydrolysis in cortical slices could not be mediated by one of these receptor subtypes. Also consistent with the hypothesis that MHI is acting at a novel receptor, we found that the phosphoinositide hydrolysis response to MHI and DHPG can be distinguished based on their pharmacological profile. In general, the compounds we chose to include in the study are compounds that have been reported in the literature to have some antagonist effects on the mGluRs. However, with a few exceptions the compounds have not been tested on all of the cloned mGluR subtypes and group selectivity has been quite poor. For instance, we tested three reported group I antagonists, MCPG, 4C3HPG and AIDA. The first antagonist described to be selective for group I mGluRs relative to group II or group III was MCPG ($IC_{50} = 40\text{--}200\ \mu\text{M}$). However, it is now known that MCPG can antagonize members of both group II and group III mGluRs (for review see Conn and Pin, 1997). (S)-4C3HPG was found to be a potent competitive antagonist of mGluR1, with an IC_{50} of $10\ \mu\text{M}$ (Thomsen *et al.*, 1994b, Ferraguti *et al.*, 1994, and Hayashi *et al.*, 1994). However, (S)-4C3HPG may be a partial agonist of mGluR5 and has been found to be an agonist at group II mGluRs (Thomsen *et al.*, 1994b). More recently, AIDA ($IC_{50} = 7\ \mu\text{M}$) was found to be even more potent than these phenylglycine derivatives on mGluR1a but is inactive on mGluR2 and mGluR4 (Pellicciari *et al.*, 1995). However, its effects on mGluR5 and the other cloned mGluRs is at present unknown (for review of the selectivities of these and other compounds used in this study, see Conn and Pin, 1997). Recently, a new antagonist has been reported that potently blocks group II mGluRs. This compound, LY341495, inhibits glutamate's actions on human mGluR2 and mGluR3 with IC_{50} s of 34 and 11 nM, respectively, and has no detectable effects at iGluRs (Ornstein *et al.*, 1996). We report that LY341495 also blocks the phosphoinositide hydrolysis response elicited by activation of mGluR1a and mGluR5a, albeit at lower potencies than at group II mGluRs.

Interestingly, LY341495 has no effect on the phosphoinositide hydrolysis response to MHI in cortical slices. Similarly, MCPG, a previously characterized antagonist of group I mGluRs, was ineffective in blocking the phosphoinositide hydrolysis response to MHI in cortical slices. In contrast to their effects on the response to MHI, both MCPG and LY341495 blocked the phosphoinositide hydrolysis response to the group I mGluR agonist, DHPG, in cortical slices. Taken together, these data provide strong evidence that MHI elicits a phosphoinositide hydrolysis response in rat brain by activation of a novel receptor that is distinct from the known mGluRs. Coupled with evidence that MHI does not activate receptors of other known neurotransmitter systems, it is likely that MHI is acting at an as of yet uncloned and uni-

identified acidic amino acid receptor coupled to phosphoinositide hydrolysis.

It is interesting to note that, in a previous study, we found that BrHI had no effect on the inhibition or potentiation of cAMP in rat cortical slices. Further, BrHI also had no effect on PLD activity. In our study, however, although maximal concentrations of both MHI and BrHI have no significant effects on mGluR2, both glutamate analogs have slight but significant effects on group III mGluRs stably expressed in BHK cells. This apparent discrepancy between the effect of these compounds on brain slices and the cloned receptors in expression systems could be due to lower levels of expression of group III receptors in slices compared to receptor expression levels in BHK cells.

Although the data reported here are suggestive that MHI increases phosphoinositide hydrolysis by activation of a novel mGluR, there are possible alternative explanations for these data. It is unlikely that a group II mGluR (mGluR2 or mGluR3) mediates this response because MHI does not activate mGluR2 and the selective group II agonist, DCG-IV, which potently activates both mGluR2 and mGluR3 (Hayashi *et al.*, 1993) was not able to activate phosphoinositide hydrolysis at mGluR1a expressing cells (Hayashi *et al.*, 1993) or in rat brain slices (Nicoletti *et al.*, 1993, Schoepp *et al.*, 1996). However, we did find that although MHI is inactive at group I mGluRs, this compound is a partial agonist at group III mGluRs. Thus, it is possible that the phosphoinositide hydrolysis response to MHI may be mediated by activation of group III mGluRs. However, this is unlikely in light of a large number of previous studies revealing that group III mGluRs do not couple to phosphoinositide hydrolysis in expression systems (Conn and Pin, 1997) and that selective agonists of group III mGluRs do not activate phosphoinositide hydrolysis in rat brain slices (Schoepp *et al.*, 1995). Another possibility is that MHI induces phosphoinositide hydrolysis by way of its known agonist activity at α -amino-3-hydroxy-5-methyl-isoxazole 4-propionate subtype of ionotropic glutamate receptor (Krogsgaard-Larsen *et al.*, 1980). However, all of the studies with MHI were performed in the presence of high concentrations of ionotropic glutamate receptor antagonists (Chung *et al.*, 1994) that completely block α -amino-3-hydroxy-5-methyl-isoxazole 4-propionate receptor-mediated inward currents induced by 1 mM MHI (unpublished results). Thus, the response to MHI is not likely mediated by activation of α -amino-3-hydroxy-5-methyl-isoxazole 4-propionate receptors. A final possibility is that MHI in some way induces release of another neurotransmitter that then activates phosphoinositide hydrolysis. However, we found that the response to MHI is not blocked by tetrodotoxin or by antagonists of a variety of receptors that are known to couple to activation of phosphoinositide hydrolysis in cortical slices. These include antagonists of muscarinic, α -adrenergic, H1 histaminergic, 5HT₂ serotonergic receptors and group I mGluRs. Although it is not possible to entirely rule out the possibility that MHI induces release of another neurotransmitter, these studies suggest that the effect of MHI is not dependent on increased cell firing or activation of any of the major known phosphoinositide hydrolysis-linked receptors in cortical slices.

In summary, our findings suggest that MHI activates phosphoinositide hydrolysis in rat cortical slices by activation of a novel mGluR that is distinct from the previously

cloned mGluR subtypes. In future studies it will be important to establish the molecular identity of this putative mGluR. MHI may provide an excellent tool for cloning of this receptor using an expression system suitable for phosphoinositide hydrolysis-linked receptors, such as *Xenopus* oocytes.

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