BAY36-7620: a potent non-competitive mGlu1 receptor antagonist with inverse agonist activity

Carroll Fiona Y. [1] **, Stolle Andreas** [2] **, Beart Philip M.** [3] **, Voerste Arnd** [2] **, Brabet Isabelle** [1] **, Mauler Frank** [2] **, Joly Cécile** [1] **, Antonicek Horst** [2] **, Bockaert Joël** [1 4] **, Müller Thomas** [2] **, Pin Jean-Philippe** [1] **, Prézeau Laurent** [1 *]

[1] *IGF, Institut de génomique fonctionnelle CNRS : UMR5203, INSERM : U661, Université Montpellier I, Université Montpellier II - Sciences et Techniques du Languedoc, 141, Rue de la Cardonille 34094 MONTPELLIER CEDEX 5,FR*

[2] *Department of Neurosciences Bayer, DE*

[3] *Department of Pharmacology Monash University, Victoria,AU*

[4] *LGF, Laboratoire de génomique fonctionnelle CNRS : UPR2580, 141, Rue de la Cardonille 34094 MONTPELLIER CEDEX 5,FR*

* Correspondence should be adressed to: Laurent Prézeau <prezeau@montp.inserm.fr>

Abstract

L-glutamate (Glu) activates at least eight different G protein-coupled receptors, the metabotropic glutamate (mGlu) receptors, which mostly act as regulators of synaptic transmission. These receptors consist of two domains: an extracellular one where agonists bind, and a transmembrane heptahelix region involved in G-protein activation. Although new mGlu receptor agonists and antagonists have been described, few are selective for a single mGlu subtype. Here, we have examined the effects of a novel compound BAY36-7620 [(3aS,6aS)-6a-Naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[c]furan-1-on], on mGlu receptors (mGlu1-8), transiently expressed in HEK 293 cells. BAY36-7620 is a potent (IC_{50} = 0.16 M) and selective antagonist at mGlu1 receptors and inhibits >60% of mGlu1a receptor constitutive activity (IC50 = 0.38 M). BAY36-7620 is therefore the first described mGlu1 receptor inverse agonist. To address the mechanism of action of BAY36-7620, Glu dose-response curves were performed in the presence of increasing concentrations of BAY36-7620. The results show that BAY36-7620 largely decreases the maximal effect of Glu. Moreover, BAY36-7620 did not displace the [^{3}H]quisqualate binding from the Glu-binding pocket., further indicating that BAY36-7620 is a non-competitive mGlu1 antagonist. We then looked for its site of action. Studies of chimeric receptors containing regions of mGlu1, and regions of DmGluA, mGlu2 or mGlu5, revealed that the transmembrane region of mGlu1 is necessary for activity of BAY36-7620. Transmembrane helices 4–7 are shown to play a critical role in the selectivity of BAY36-7620. This specific site of action of BAY36-7620 differs from that of competitive antagonists, and indicates that the transmembrane region plays a pivotal role in the agonist-independent activity of this receptor. BAY36-7620 will be useful to further delineate the functional importance of the mGlu1 receptor, including its putative agonist-independent activity.

MESH Keywords Animals ; Cells, Cultured ; Humans ; Inositol Phosphates ; metabolism ; Membrane Proteins ; drug effects ; metabolism ; Naphthalenes ; pharmacology ; Rats ; Receptors, Metabotropic Glutamate ; agonists ; metabolism ; Transfection

Introduction

Metabotropic glutamate (mGlu) receptors are G protein-coupled receptors (GPCRs) which act predominantly as modulators of synaptic transmission. As such, they play a role in many physiological and pathophysiological processes (Conn and Pin, 1997). The mGlu receptors are members of the family 3 heptahelical GPCRs, which also comprise the calcium-sensing receptor (CaSR), the GABA-B receptors and some putative pheromone and taste receptors (Bockaert and Pin, 1999). One specific feature of these receptors was their large extracellular domain (ECD) proposed to contain the agonist binding site (Bessis et al., 2000; Hammerland et al., 1999; Han and Hampson, 1999; Malitschek et al., 1999; O'Hara et al., 1993; Okamoto et al., 1998), an idea recently confirmed by the resolution of the crystal structure of the mGlu1 ECD (Kunishima et al., 2000).

Eight different mGlu receptor genes have been identified to date, and, according to their sequence similarity, have been classified into three groups (Conn and Pin, 1997). Group I (mGlu1 and mGlu5 receptors) are coupled to the activation of phospholipase C, whereas group II (mGlu2 and mGlu3 receptors) and group III (mGlu4, mGlu6, mGlu7 and mGlu8 receptors) members are all negatively coupled to adenylyl cyclase in heterologous expression systems. Many of these receptors exist as different forms generated by alternative splicing (Pin et al., 1999). For mGlu1 receptors, several splice variants have been identified, that vary in the length of their intracellular C- terminal tail, with the mGlu1a variant containing the largest C-terminal tail (360 residues), with the last 313 residues of which are replaced by 11-26 residues in the other variants.

The existence of numerous mGlu receptor subtypes and splice variants makes the need for highly selective and potent agonists and antagonists crucial for physiological and therapeutical studies of mGlu receptors. Indeed, within recent years, a number of ligands selective for the individual groups of mGlu receptors have been developed (Pin et al., 1999; Schoepp et al., 1999). However, the high degree of conservation of the agonist binding pocket between mGlu receptors from the same group (Parmentier et al., 2000) is such that only very few subtype selective compounds acting on this site have been identified (Pin et al., 1999; Schoepp et al., 1999). Moreover, these compounds display a very low affinity that precludes their usefulness in identifying the physiological roles of these receptors. Recently, selective non-competitive antagonists have been identified for mGlu1 (CPCCOEt) and mGlu5 (MPEP) (Hermans et al., 1998; Litschig et al., 1999; Pagano et al., 2000). Notably, the structures of both compounds are unrelated to that of Glu (Fig. 1), and both were found to interact directly within the transmembrane (TM) region of the receptors (Litschig et al., 1999; Pagano et al., 2000). Thus, mGlu receptors can be specifically regulated by compounds, with diverse structures, acting on sites other than the Glu binding site.

An increasing number of constitutively active receptors – i.e. active in the absence of an agonist - have been described, especially among the rhodopsin-like receptors. Thus, it has been proposed that GPCRs naturally oscillate between active and inactive states in the absence of agonist binding (Cotecchia et al., 1990; Kjelsberg et al., 1992; Leff, 1995; Samama et al., 1993). As such, agonists stabilize the active state, whereas antagonists do not. Among the antagonists, those that stabilize an inactive state can inhibit not only the agonist-induced activation of the receptor, but also its constitutive activity. Such antagonists are called inverse agonists. For most constitutively active GPCRs characterized to date, numerous competitive antagonists display inverse agonist activity. Such compounds are very useful for the understanding of the possible physiological relevance of GPCR constitutive activity (Arvanitakis et al., 1998; Barker et al., 1994; Bond et al., 1995; Claeysen et al., 1999).

We have previously shown that mGlu5 as well as mGlu1a receptors display agonist-independent activity (Joly et al., 1995; Prezeau et al., 1996). However, none of the described competitive antagonists for mGlu1 or mGlu5 receptors, which act in the ECD, have inverse agonist properties (Prezeau et al., 1996). The first mGlu receptor inverse agonist described was MPEP, a non-competitive mGlu5 receptor antagonist acting in the TM region rather than in the ECD (Pagano et al., 2000). However, CPCCOEt, a selective mGlu1 non-competitive antagonist also interacting within the TM region, did not show any significant inverse agonist activity (Litschig et al., 1999).

A new compound, BAY36-7620 ([(3aS,6aS)-6a-Naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[c]furan-1-on]; Fig. 1), has recently been found to inhibit the Glu-induced activation of PLC-coupled mGlu receptors in cultured neurons (described in abstract form; Mü ller et al., 2000). Here, we show that BAY36-7620 is a selective non-competitive mGlu1 antagonist acting within the TM region. BAY36-7620 is also shown to inhibit the agonist-independent activity of mGlu1a receptor, being therefore the first inverse agonist of this receptor subtype.

Material and Methods

Materials

Glutamate and bicinchoninic acid were purchased from SIGMA-ALDRICH (L'ïsle D'abeau Chesne, France). 2-(3′-carboxybicyclo[1.1.1] pentyl)-glycine (CBPG), S-α-methyl-4-carboxyphenylglycine (MCPG) and quisqualate were purchased from Tocris Cookson (Bristol, U.K.). Glutamate pyruvate transaminase was purchased from Boehringer Mannheim (Meylan, France). [^{3}H]-Quisqualate ([^{3}H]QA, 25 Ci/mmole) and PCS scintillant were purchased from Amersham (Saclay, France). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany). Whatmann QF/C filters were purchased from Whatmann (Gladstone, England). BAY36-7620 has been synthesized by BAYER as described (Müller et al., 2000). Fetal bovine serum, culture media, and other solutions used for cell culture were purchased from GIBCO-BRL-Life Technologies, Inc. (Cergy Pontoise, France). [^{3}H]-myo-inositol (23.4 Ci/mol) was purchased from NEN (Paris, France). All other reagents used were of molecular or analytical grade where appropriate.

Constructs

The expression plasmids containing the rat mGlu1a, mGlu2, mGlu3, mGlu4a, mGlu5a and mGlu6, mGlu7a and mGlu8a cDNAs have been previously described (Brabet et al., 1998; Gomeza et al., 1996; Parmentier et al., 1998). The chimera mGlu1/DmGluRA construct has been described previously (Parmentier et al., 1998). For generation of chimeras between mGlu1 and mGlu2, a silent EcoRV site was generated in each of mGlu1 and mGlu2 at positions Asp590-Ile591 and Asp565-Ile566 respectively. This site was then used to exchange the extracellular domains of mGlu1 and mGlu2 by subcloning of the fragments EcoRI-EcoRV or EcoRV-XbaI. For construction of chimeras between mGlu1 and mGlu5 the following new restriction sites were generated. For mGlu1, an EcoRV site was created at position Gly329-Tyr330-Glu331 changing this to the corresponding sequence from mGlu5, Gly315-Tyr316-Gln317. A silent NheI site was also generated at Leu604-Leu605-Ala606 corresponding to the identical sequence in mGlu5 (Leu590-Leu591-Ala592) and where necessary, a silent mutation removed the EcoRI site at position Glu121-Phe122. For mGlu5, a silent BglII restriction site was created at position Lys678-Ile679-Cys680

corresponding with an identical sequence in mGlu1 (Lys692-Ile693-Cys694). These new restriction sites were then used to generate the constructs given in Fig. 7A as follows: the EcoRV site was used to exchange the first half of the extracellular domain (chimeras A & B), the NheI site was used to exchange the extracellular domain (chimeras C& D), and exchange of the extracellular domain plus the first three TMs (chimera E) was performed using the Bgl II site located in the sequence coding for the i2 loop. For chimera F, we subcloned the fragment Nhe I – Bgl II of mGlu5 into mGlu1.

Culture and transfection of HEK 293 cells

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and transfected by electroporation as previously described (Gomeza et al., 1996). Briefly, electroporation was carried out in a total volume of 300 µl with 10 µg carrier DNA, plasmid DNA containing mGlu1a (0.3 µg), mGlu5a (0.3 µg), mGlu2 (2 µg), mGlu3 (2 µg), mGlu4a (5 µg), mGlu6 (5 µg), mGlu7a (5 µg) or mGlu8a (5µg) and 10 million cells (Brabet et al., 1998). Group-I mGlu receptors naturally couple to phospholipase C (PLC), but the group-II and -III subtypes do not. Thus, to enable coupling of the group II and III mGlu receptors to PLC, the receptors were coexpressed with the chimeric G-protein α subunit Gqi9 in which the carboxyl terminal 9 residues of Gq are replaced by those of Gi2 (Conklin et al., 1993; Gomeza et al., 1996; Parmentier et al., 1998). mGlu6 was however co-expressed with the non-selective PLC-activating G15 subunit (Offermanns and Simon, 1995). The pharmacological profiles of mGlu2 and mGlu4 determined using this method of coupling to PLC have been shown to be identical to those determined by measuring the inhibition of cAMP formation (Gomeza et al., 1996). As Glu concentration in the culture medium profoundly affects the functioning of mGlu5a and mGlu3 receptors, these receptors were co-expressed with the high affinity Glu transporter EAAC1 (Kanai and Hediger, 1992).

Determination of accumulation of inositol phosphates (IP)

Determination of IP accumulation in transfected cells was performed (Brabet et al., 1998) after labeling the cells overnight with [^{3}H] -myo-inositol (23.4 Ci/mol). The cells were pre-incubated for 1 hour with the Glu degrading enzyme glutamate pyruvate transaminase (1U/ml) and 2 mM pyruvate to avoid the possible action of Glu released from the cells. The stimulation was then conducted for 30 min in a medium containing 10 mM LiCl, glutamate pyruvate transaminase, pyruvate and the indicated concentration of agonist. Results are expressed as the ratio between the radioactivity of collected in the IPs fraction over the radioactivity recovered from the solubilized cellular membranes. The use of this ratio over IP formation alone, allows for greater homogeneity in the data, as this reduces any variation in the raw data attributable to differences in cell number in individual wells. The normalized IP formation were determined as a percentage of IP formation ratio compared to that obtained with the submaximal Glu concentration used in the described experiments and referred as 100 %.

Membrane preparation and [^{3}H]-Quisqualate Binding Assay

[^{3}H]QA binding was performed in HEK293 cells cultured and transiently transfected with mGlu1as receptor as described above. Membranes were recovered 24 hours after transfection in KREBS-Tris buffer (Tris 20mM, NaCl 118mM, KH_2PO_4 1.2mm, $MgSO_4$ 1.2 mM, KCl 4.7mM, $CaCl_2$ 1.8mM, glucose 5.6mM, pH 7.4), homogenized, pooled and centrifuged at 40,000g for 20 min. The resulting pellet was resuspended in the same buffer, homogenized and stored as pellets at −20°C until use (< 1 month). Protein levels were determined using a bicinchoninic acid assay (Smith et al., 1985) with bovine serum albumin as a standard.

Binding conditions used were communicated by Dr. C. Romano (personal communication) and performed and modified as follows. Binding experiments were carried out in a buffer containing HEPES 40mM and $CaCl_2$ 2.5mM, adjusted to pH 7.4 with NaOH. Membrane pellets were resuspended in the above buffer plus protease inhibitor cocktail and aliquots of 50µg protein were incubated in the presence of [^{3}H] QA (specific activity 25Ci/mmol), in a final volume of 100µl at room temperature, for one hour. Competition experiments were performed with 600nM [^{3}H]QA and varying concentrations of cold QA and BAY36-7620. Incubation was terminated by rapid filtration through Whatmann QF/C filters, (pre-soaked for 45 min in 3% powdered milk), using a Brandel Harvester. Filters were rinsed in HEPES/$CaCl_2$ buffer and counted in 3ml PCS scintillant. Non-specific binding was determined in the presence of 1mM Glu.

Data presentation and Statistics

Curves were fitted with Kaleidagraph software using the equation $y = [(ymax - ymin)/(1 + (x/EC_{50})^{nH})) + ymin$ where the EC_{50} is the concentration of the compound necessary to obtain 50% of the maximal effect and nH is the Hill coefficient. Statistical analysis was performed using Microsoft™ Excel software. Where appropriate one way analysis of variance were performed followed by post-hoc Students T-Test with $\alpha = 0.05$. All t-tests used 2-way critical values, and only the pertinent values are given.

Results

BAY36-7620 is a specific mGlu1 receptor antagonist

BAY36-7620 (Fig. 1), has been found to antagonize the action of group-I mGlu receptor agonists on cerebellar granule neurons maintained in culture (Müller et al., 2000), suggesting that this new compound is an mGlu1 receptor antagonist as this receptor is the predominant group-I mGlu receptor present in these cells (Prezeau et al., 1994).

The effect of BAY36-7620 on the production of inositol phosphates (IP) following receptor stimulation of phospholipase C, was first examined in cells transiently expressing one of the group-I mGlu receptors, mGlu1a or mGlu5a. BAY36-7620 did not activate these receptors at concentrations up to 10 µM (Fig. 2A). To test for an antagonistic effect of BAY36-7620 on mGlu1 or mGlu5 receptors, cells were stimulated with a Glu concentration around its EC_{50} value (1 µM for mGlu1a & 5 µM for mGlu5a), in the presence of BAY36-7620, applied at a concentration of 0.1 or 10 µM. As shown in Figure 2A, a strong inhibition of the Glu effect (61 ± 3 % inhibition) was observed with 0.1 µM BAY36-7620 and a total inhibition was observed with 10 µM BAY36-7620 on mGlu1 receptors whereas no effect was seen on mGlu5 receptors. The antagonist effect of BAY36-7620 on mGlu1a receptors was concentration-dependent with an IC_{50} of 0.16 ± 0.01 µM and a hill coefficient of 1.30 ± 0.11 (Fig. 2B and Table 1).

To further characterize the selectivity of action of BAY36-7620, its effect on all other mGlu receptor subtypes was examined. The same index of receptor activation as for group-I mGluRs –i.e. IP formation - was used. To that aim, the Gi/o-coupled group-II and –III mGluRs were co-expressed with either a chimeric Gαqi protein, or the ubiquitous Gα15 protein, as previously described (see the Material and Methods section and (Brabet et al., 1998)). BAY36-7620 (10 µM) did not stimulate IP formation in cells expressing mGlu2, 3, 4, 6, or 7 receptors (Fig. 3). A small response was detected in cells expressing mGlu8 receptor, equal to 36.9 ± 13.3 % of the effect obtained with 1 mM Glu on mGlu1 receptor. This effect was concentration-dependent with an EC_{50} of 15.8 ± 2.6 µM, 100 fold higher than the IC_{50} determined on mGlu1 receptor (data not shown). However, we noted that the slope of the curve was very steep, the Hill coefficient being 5.32 ± 0.59, suggesting a complex effect of BAY36-7620 on this receptor subtype. BAY36-7620 may be an allosteric activator, which facilitates the action of low residual Glu in the medium. When tested as an antagonist, BAY36-7620 was found not to inhibit the Glu-induced activation of mGlu2, 3, 4, 6, 7, or 8 receptors (Fig. 3). Together, these results indicate that BAY36-7620 is a potent and specific mGlu1 receptor antagonist.

BAY36-7620 is an inverse agonist of mGlu1a receptors

Numerous antagonists at family 1 GPCRs inhibit constitutive activity of either wild-type or mutated receptors, and are thus referred to as inverse agonists (Arvanitakis et al., 1998). However, no known competitive group-I mGlu receptor antagonists nor the non-competitive antagonist CPCCOEt have been found to inhibit the constitutive activity of these receptors (Litschig et al., 1999; Prezeau et al., 1996). However, we decided to ascertain whether this novel antagonist had observable inverse agonist activity.

Indeed, BAY36-7620 significantly inhibited the basal production of IP by approximately 36% at 10 µM (Fig. 4A; $t_{(df=23)} = 2.9$, $p<0.05$). Co-expression of the Gαq subunit of G-protein with mGlu1 receptor boosts levels of constitutive activity (Parmentier et al., 1998) enabling an easier analysis of inverse agonist activity. Under these conditions BAY36-7620 inhibited the basal PLC activity by approximately 44% at 10 µ M in these mGlu1a receptor expressing cells (Fig. 4B; $t_{(df=4)} = 5.58$, $p<0.05$). Moreover this inhibition was concentration-dependent with an IC_{50} of 0.38 ± 0.19 µM, close to that obtained for the inhibition of Glu-stimulated IP production (Fig. 4C and Table 1). Moreover, under both conditions, when incubated in the presence of Glu, BAY36-7620 decreased the IP production to a level significantly lower than the basal level ($t_{(df=23)} = 2.3$, $p<0.05$ and $t_{(df=5)} = 3.9$, $p<0.05$ in the presence of Gαq), indicating that BAY36-7620 inhibited not only the activation of the receptor by Glu, but also its basal activity. As a control, MCPG (3mM), a non-specific competitive mGlu1 receptor antagonist whilst significantly inhibiting the Glu-induced stimulation of IP ($t_{(df=16)} = 2.4$, $p<0.05$), did not inhibit the constitutive activity of mGlu1a receptors (Fig. 4B). These data demonstrate that BAY36-7620 is a potent inverse agonist at mGlu1a receptor.

BAY36-7620 is a non-competitive mGlu1 receptor antagonist

To ascertain the mechanism of antagonism by BAY36-7620, concentration-response curves for Glu were generated in the presence of 0, 0.1, 1.0 and 10 µM BAY36-7620 (Fig. 5). The maximal effect of Glu decreased with high concentrations of BAY36-7620 from 25.2 ± 1.55 %, in the absence of BAY36-7620, to 26.3 ± 2.04 %, 20.0 ± 3.07 % and 10.2 ± 2.93 % in the presence of 0.1, 1 and 10 µM BAY36-7620 respectively, indicating that BAY36-7620 did not inhibit the receptor in a competitive manner. We observed that the EC_{50} of Glu also increased as the concentration of BAY36-7620 increased, whereas the Hill coefficient of the Glu concentration-response curves was not affected by increasing the concentration of BAY36-7620.

Further confirmation of the non-competitive nature of BAY36-7620 was sought through analysis of binding of [^{3}H]QA, a potent group-I mGlu receptor agonist known to bind at the Glu binding site (Schoepp et al., 1999), to HEK293 cell membranes expressing mGlu1a. As shown in Fig. 6, BAY36-7620 was not effective in displacing the binding of 600nM [^{3}H]QA on mGlu1, up to 100 μM ($F_{(2,26)} = 0.37$, $p>0.05$), whereas cold QA did so (the specific binding was 45 ± 6 % of the total binding of [^{3}H]QA). Moreover, the capability of cold QA to displace [^{3}H]QA was not modified by the presence of 10 μM of BAY36-7620, since its Ki values were 109 nM and 85 nM, in the absence and in the presence of BAY36-7620 respectively ($F_{(2,11)} = 7.4$, $p<0.05$; Fig. 6). These data are consistent with BAY36-7620 acting at a site different from the Glu binding site.

BAY36-7620 is likely to interact within the transmembrane region of mGlu1 receptor

The previously described non-competitive group-I mGlu receptor antagonists, CPCCOEt and MPEP, have been reported to bind within the TM region of mGlu1 and mGlu5 receptors respectively (Litschig et al., 1999; Pagano et al., 2000). We therefore examined the effect of BAY36-7620 on various chimeric receptors in which the ECD had been swapped between mGlu1 and the drosophila receptor DmGluA, or between mGlu1 and mGlu2. As shown in Fig. 7, BAY36-7620 did not inhibit the action of Glu on receptors possessing the ECD of mGlu1 receptor and the TM region of either DmGluA or mGlu2 receptors. However, BAY36-7620 fully antagonized the action of Glu on the chimeric mGlu2/1 receptor containing the TM region of mGlu1 receptor and ECD of mGlu2 receptor. On this chimeric receptor, the inhibitory effect of BAY36-7620 was also concentration-dependent with an IC_{50} value of 0.14 ± 0.05 μM, not notably different from that measured on the wild-type mGlu1 receptor (Table 1). Taken together, these data revealed a crucial role played by the TM region of the mGlu1 receptor for the action of BAY36-7620 on this receptor subtype.

To further identify the molecular determinants controlling the specificity of BAY36-7620, a series of chimeric receptors possessing various parts of the ECD and TM regions of the mGlu1 and mGlu5 receptor subtypes were constructed. A schematic representation of these chimeric receptors is shown in Fig. 8A. Analysis of the action of BAY36-7620 on these chimeric receptors confirmed that the antagonist action of this compound on Glu-induced IP stimulation is effective on chimeric receptors A and C containing the TM region of mGlu1 receptor (Fig. 8B, chimeras A, $t_{(df=7)} = 10.3$, $p<0.05$; & C, $t_{(df=2)} = 19.7$, $p<0.05$). Conversely, for the chimeric receptors B and D, which possess the mGlu5 receptor TM region, BAY36-7620 was ineffective in inhibiting the Glu-induced activation of the receptors (Fig. 8B, chimeras B, $t_{(df=3)} = 1.23$, $p>0.05$; & D, $t_{(df=3)} = 1.14$, $p>0.05$). Moreover, the construct F possessing the last four transmembrane helices (TM4-7) of mGlu1 receptor was still responsive to BAY36-7620 (Fig. 8B, chimera F; $t_{(df=7)} = 7.98$, $p<0.05$), with a potency only three times lower than that measured on the wild-type mGlu1 receptor (Table 1). Indeed, the potency of BAY36-7620 on chimeras C and F is close to the potency on mGlu1 receptor (Fig. 8C, Table 1). In contrast, the chimera E containing the first three TMs of mGlu1 receptor and the last four of mGlu5 receptor (Fig. 8B, chimera E; $t_{(df=2)} = 3.23$, $p>0.05$) is not sensitive to antagonism by BAY36-7620. These data indicate that residues crucial for the specific action of BAY36-7620 on mGlu1 over mGlu5 receptors are located within the last four TMs.

Discussion

In the present study, we show that a new compound, BAY36-7620, that shares no structural similarity with Glu, is a specific mGlu1 receptor non-competitive antagonist. This compound also inhibits at least part of the agonist-independent activity of the mGlu1a receptor, and is as such, the first described inverse agonist for mGlu1 receptors. Finally, our data show that the TM region is critical for the specific action of this compound on the mGlu1 receptor, indicating that, like the other non-competitive mGlu receptor antagonists recently identified, BAY36-7620 is likely to interact within the TM region of this receptor subtype.

A few years ago, the demonstration that not only mutated, but also wild-type GPCRs could display agonist-independent activity changed views on how these heptahelix receptors function (Arvanitakis et al., 1998). Indeed, it is thought that these proteins can oscillate between active and inactive conformations. Accordingly, agonists activating these receptors were proposed to preferentially bind to, and stabilize, the active conformation of the receptor. Conversely, most antagonists were found to inhibit the agonist-independent activity of the receptors, most likely by interacting preferentially with an inactive conformation of the receptor. Indeed, only few compounds do not affect the natural equilibrium of the receptor and as such are pure neutral antagonists. Such a tendency of heptahelix receptors to undergo agonist-independent activity has been extensively studied within the family 1, rhodopsin-like receptors (Arvanitakis et al., 1998; Chidiac et al., 1994; Lefkowitz et al., 1993). However, agonist-independent activity of family 3 mGlu-like receptors has also been described, both for wild-type and mutated receptors (Brown and Hebert, 1997; Joly et al., 1995; Prezeau et al., 1996; Jensen et al., 2000). In the case of the CaSR, such mutations leading to constitutively active receptors are likely to be responsible for familial autosomal dominant hypocalcaemia (Brown and Hebert, 1997).

Most of the family 3 receptors are constituted of two large domains, the ECD and the heptahelical TM region which is responsible for the coupling to the G-protein (Bockaert and Pin, 1999). The ECD, that contains the ligand binding site, can exist as at least two different forms, an inactive open one, and an active closed one, as suggested by several mutagenesis and modeling studies (see for example Bessis et al., 2000; Galvez et al, 2000) and recently demonstrated by the determination of the crystal structure of the ECD of mGlu1 receptor (Kunishima et al., 2000). Then, it was thougth that the constitutive activity of the family 3 receptor could be due to the spontaneous closure of the ECD. Indeed, some studies showed that mutations in the ECD of the CaSR resulted in constitutively active receptor (Jensen et al. 2000). But the natural constitutive activity of mGlu1 and mGlu5 receptors does not result from the equilibrium of the ECD between an active and an inactive state. First, the six known competitive antagonists (ABHD-I, bromo-homo-ibotenate, MCPG, 4CPG, 4C3HPG and ACPT-III, (see Joly et al., 1995; Prezeau et al., 1996) and Parmentier and Pin, unpublished data) that bind to the ECD of mGlu1 and mGlu5 receptors, do not block their natural constitutive activity. Indeed, all these competitive antagonists behave as neutral antagonists. Second, only non-competitive antagonists (such as MPEP and BAY36-7620), acting directly in the TM region, are able to block the constitutive activity. This suggests that the agonist-independent activity of mGlu1 receptors can not be blocked by acting directly in the agonist binding pocket. This is in contrast to what has been demonstrated for many Rhodopsin-like receptors, for which competitive antagonists behave as inverse agonists. Our finding that BAY36-7620 has inverse agonist property on mGlu1a receptor is therefore of interest to increase understanding of the specific molecular mechanisms responsible for the agonist-independent activity of the family 3 receptors.

In contrast to all competitive Glu-like antagonists, we found that BAY36-7620 interacts in a specific site distinct from that of Glu. Indeed, BAY36-7620 did not displace [^{3}H]QA from its binding site, nor did it affect the affinity of cold QA. Moreover, the antagonist action of BAY36-7620 was found not to be competitive, since the maximal Glu-induced activation of mGlu1 receptor was decreased in the presence of high concentration of BAY36-7620. At low concentrations, BAY36-7620 did not decrease the maximal effect of Glu and did decrease significantly the EC_{50} value of Glu. Such an effect can easily be explained by the presence of spare receptors, as expected using transient expression in HEK 293 cells. Indeed, a similar decrease in the glutamate EC_{50} value using a low concentration of a non-competitive antagonist has also been reported with CPCCOEt (Hermans et al., 1998).

Our data using chimeric mGlu receptors revealed that the TM region of mGlu1 was necessary for the inhibitory action of BAY36-7620. Moreover, its apparent affinity was the same whether the ECD was that of either mGlu5, mGlu2 or DmGluA receptors even though these later two ECDs share only 41% sequence identity with that of mGlu1 receptor. Within the TM regions, we found that TM4 to TM7 of mGlu1 receptor were sufficient to create a BAY36-7620 site in mGlu5 receptor. This shows that in contrast to what has been observed for the selectivity of action of MPEP (Pagano et al., 2000), TM3 does not play a role in the selective recognition of BAY36-7620 in the mGlu1 receptor. Amongst the TM4 to 7, TM6 is identical in all mGlu receptors, and as such cannot play a role in the specific recognition of BAY36-7620, but a few residues are different between mGlu1 and mGlu5 receptor in TM4, TM5 and TM7. Preliminary experiments have revealed that indeed, as observed with the other mGlu1 selective non-competitive antagonist, CPCCOEt (Litschig et al., 1999), TM7 of mGlu1 appears critical for the action of BAY36-7620 (Carroll, Kuhn, Pin and Prézeau, unpublished data). It has recently been proposed that CPCCOEt and the mGlu5 selective non-competitive antagonist MPEP interact within a similar cavity found in both mGlu1 and mGlu5 receptors (Litschig et al., 1999; Pagano et al., 2000). Indeed, it has been shown that only few residues within this binding pocket are responsible for the high selectivity of these two molecules. According to our data, it is likely that BAY36-7620 binds in the same cavity as these other non-competitive mGlu receptor antagonists. Although more work is necessary to further characterize the BAY36-7620 binding site, our data are sufficient to demonstrate the pivotal role played by the TM region of mGlu1 receptor for the antagonistic action of BAY36-7620.

Of interest, not only BAY36-7620, but also MPEP have been found to be inverse agonists. Moreover, although no inverse agonist activity of CPCCOEt could be detected on native mGlu1 receptors expressed alone (Litschig et al., 1999), we recently found that this compound is able to significantly inhibit 10-15% of the agonist-independent activity of mGlu1 receptors boosted by co-expression with the Gαq subunit (F. Carroll, unpublished data), indicating that CPCCOEt is a very partial inverse agonist at mGlu1 receptors. Taken together, these data show that amongst all the non-competitive antagonists which have been shown to interact within the TM region of either mGlu1 or mGlu5 receptors, all have inverse agonist activity. This is in contrast with all competitive antagonists known to bind on the ECD of these receptors. Accordingly, we propose that the natural constitutive activities of mGlu1 and mGlu5 receptors originate more from their TM region than from their ECD. Indeed, it may be possible that the TM region, like that of the rhodopsin-like GPCRs, oscillates between active and inactive states, even when the ECD remains in an inactive (open) conformation. The binding of the non-competitive antagonists within the TM region would stabilize an inactive state as observed with the rhodopsin-like receptors. In contrast, the binding of a competitive antagonist within the ECD would prevent the binding of an agonist, and maintain it in an inactive open state, but would not be able to prevent the equilibrium between active and inactive states of the TM region of these receptors.

Taken together, our study identified a new highly selective mGlu1 receptor antagonist that will be useful to further elucidate the action of this receptor subtype in the brain. Moreover, our data showed that BAY 36-7620 is an inverse agonist on mGlu1. As the constitutive activity of mGlu1 is under the control of alternative splicing (Prezeau et al., 1996), this compound will be useful to help discriminate between some of the actions of mGlu1 variants. Our study further documents the observation that antagonists acting within the TM region of mGlu1 and mGlu5 receptors can inhibit their agonist-independent activity. Such an activity of these compounds shed some light on the specific role of the TM region of these receptors in their natural constitutive activity. Moreover, such an activity of BAY36-7620 and MPEP will be useful to elucidate the possible physiological relevance of the mGlu receptor constitutive activity. Of interest, inverse agonists of several family 1 receptors have been reported to have specific properties not shared by neutral antagonists, as for example in the case of 5HT2C (Barker et al., 1994) or 5HT1A (Albert et al., 1999) receptors.

Ackowledgements:

This work was supported by grants from the CNRS (J.B.), Bayer company (France and Germany) (J.B.), the Fondation pour la Recherche Médicale (J.B.), the "Action Incitative Physique et Chimie du Vivant" (PCV00-134) from the CNRS (J.P.P.), the program "Molécules et Cibles Thérapeutiques" from INSERM and CNRS (J.P.P.), the association Retina France (J.P.P.), by the Australian NH&MRC grant (960001) (P.M.B.). F.C. was supported by an INSERM/NH&MRC exchange fellowship (997012).

The authors wish to thank Dr. M.L. Parmentier (CNRS UPR-9023, Montpellier, France) for constructive discussion, and Drs. C. Romano (Washington University, St Louis, USA) and R. Kuhn (Novartis Pharma, Basel, Switzerland) for sharing tools and protocols.

abbreviations

CPCCOEt: 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester
ECD: extracellular domain
Glu: L-glutamate
GPCR: G protein-coupled receptor
HEK: human embryonic kidney
IP: phosphoinositides
mGlu: metabotropic glutamate
MPEP: 2-Methyl-6-(phenylethynyl)pyridine
PLC: phospholipase C
QA: quisqualate
TM: transmembrane

References:

- Albert PR , Sajedi N , Lemonde S , Ghahremani MH 1999; Constitutive Gi2-dependent activation of adenylyl cyclase type II by the 5-HT1A receptor. J Biol Chem. 274: 35469- 35474
- Arvanitakis L , Geras-Raaka E , Gershengorn MC 1998; Constitutively signaling G-protein-coupled receptors and human disease. Trends in Endocrinology and Metabolism. 9: 27- 31
- Barker EL , Westphal RS , Schmidt D , Sander-Bush E 1994; Constitutively active 5-hydroxytryptamine 2C receptors reveal novel inverse agonist activity of receptor ligands. J Biol Chem. 269: 11687- 11690
- Bessis A-S , Bertrand H-O , Galvez T , De Colle C , Pin J-P , Acher F 2000; 3D-model of the extracellular domain of the type 4a metabotropic glutamate receptor: new insights into the activation process. Prot Sci.
- Bockaert J , Pin JP 1999; Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO J. 18: 1723- 9
- Bond RA , Leff P , Johnson TD , Milano CA , Rockman HA , McMinn TR , Apparsundaram S , Hyek MF , Kenakin TP , Allen LF , Lefkowitz RJ 1995; Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β2-adrenoceptor. Nature. 374: 272- 276
- Brabet I , Parmentier M-L , De Colle C , Bockaert J , Acher F , Pin J-P 1998; Comparative effect of L-CCG-I, DCG-IV and γ-carboxy-L-glutamate on all cloned metabotropic glutamate receptor subtypes. Neuropharmacology. 37: 1043- 1051
- Brown EM , Hebert SC 1997; Calcium-receptor-regulated parathyroid and renal function. Bone. 20: 303- 9
- Chidiac P , Hebert TE , Valiquette M , Dennis M , Bouvier M 1994; Inverse agonist activity of β-adrenergic antagonists. Mol Pharmacol. 45: 490- 499
- Claeysen S , Sebben M , Becamel C , Bockaert J , Dumuis A 1999; Novel brain-specific 5-HT4 receptor splice variants show marked constitutive activity: role of the C-terminal intracellular domain. Mol Pharmacol. 55: 910- 20
- Conklin BR , Farfel Z , Lustig KD , Julius D , Bourne HR 1993; Substitution of three amino acids switches receptors specificity of G_qa to that of G_ia. Nature. 363: 274- 276
- Conn P , Pin J-P 1997; Pharmacology and functions of metabotropic glutamate receptors. Ann Rev Pharmacol Toxicol. 37: 205- 237
- Cotecchia S , Exum S , Caron MG , Lefkowitz RJ 1990; Regions of the α_1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. Proc Natl Acad Sci USA. 87: 2896- 2900
- Galvez T 2000; J Biol Chem.
- Gomeza J , Mary S , Brabet I , Parmentier M-L , Restituito S , Bockaert J , Pin J-P 1996; Coupling of metabotropic glutamate receptors 2 and 4 to $G_{\alpha 15}$, $G_{\alpha 16}$ and chimeric $G_{\alpha q/i}$ proteins: characterization of new anatgonists. Mol Pharmacol. 50: 923- 930

- Hammerland LG , Krapcho KJ , Garrett JE , Alasti N , Hung BC , Simin RT , Levinthal C , Nemeth EF , Fuller FH 1999; Domains determining ligand specificity for Ca2+ receptors. Mol Pharmacol. 55: 642- 8
- Han G , Hampson DR 1999; Ligand binding to the amino-terminal domain of the mGluR4 subtype of metabotropic glutamate receptor. J Biol Chem. 274: 10008- 13
- Hermans E , Nahorski SR , Challiss RA 1998; Reversible and non-competitive antagonist profile of CPCCOEt at the human type 1alpha metabotropic glutamate receptor. Neuropharmacology. 37: 1645- 7
- Jensen A , Spalding T , Burstein E , Sheppard P , O'Hara P , Brann M , Krogsgaard-Larsen P , Braüner-Osborne H 2000; Functional importance of the Ala116-Pro136 region in the calcium-sensing receptor. J Biol; Chem. 275: 29547- 29555
- Joly C , Gomeza J , Brabet I , Curry K , Bockaert J , Pin J-P 1995; Molecular, functional and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. J Neurosci. 15: 3970- 3981
- Kanai Y , Hediger MA 1992; Primary structure and functional characterization of a high-affinity glutamate transporter [see comments]. Nature. 360: 467- 71
- Kjelsberg MA , Cotecchia S , Ostrowski J , Caron MG , Lefkowitz RJ 1992; Constitutive activation of the α1B-adrenergic receptor by all amino acid substitutions at a single site: evidence for a region which constrains receptor activation. J Biol Chem. 267: 1430- 1433
- Kunishima N , Shimada Y , Tsuji Y , Sato T , Yamamoto M , Kumasaka T , Nakanishi S , Jingami H , Morikawa K 2000; Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature. 407: 971- 977
- Leff P 1995; The two-state model of receptor activation. Trends Pharmacol Sci. 16: 89- 97
- Lefkowitz RJ , Cotecchia S , Samama P , Costa T 1993; Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. Trends Pharmacol Sci. 14: 303- 307
- Litschig S , Gasparini F , Rueegg D , Stoehr N , Flor PJ , Vranesic I , Prezeau L , Pin JP , Thomsen C , Kuhn R 1999; CPCCOEt, a noncompetitive metabotropic glutamate receptor 1 antagonist, inhibits receptor signaling without affecting glutamate binding. Mol Pharmacol. 55: 453- 61
- Malitschek B , Schweizer C , Keir M , Heid J , Froestl W , Mosbacher J , Kuhn R , Henley J , Joly C , Pin JP , Kaupmann K , Bettler B 1999; The N-terminal domain of gamma-aminobutyric Acid(B) receptors is sufficient to specify agonist and antagonist binding. Mol Pharmacol. 56: 448- 54
- Müller T , De Vry J , Schreiber R , Horvath E , Mauler F , Strolle A , Lowe D 2000; BAY 36-7620, a selective metabotropic glutamate receptor 1 antagonist with anticonvulsivant and neuroprotective properties. Soc Neurosci Abstr. 26: 1650-
- O'Hara PJ , Sheppard PO , Thogersen H , Venezia D , Haldeman BA , McGrane V , Houamed KM , Thomsen C , Gilbert TL , Mulvihill ER 1993; The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. Neuron. 11: 41- 52
- Offermanns S , Simon MI 1995; G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C. J Biol Chem. 270: 15175- 80
- Okamoto T , Sekiyama N , Otsu M , Shimada Y , Sato A , Nakanishi S , Jingami H 1998; Expression and purification of the extracellular ligand binding region of metabotropic glutamate receptor subtype 1 [In Process Citation]. J Biol Chem. 273: 13089- 96
- Pagano A , Ruegg D , Litschig S , Stoehr N , Stierlin C , Heinrich M , Floersheim P , Prezeau L , Carroll F , Pin JP , Cambria A , Vranesic I , Flor PJ , Gasparini F , Kuhn R 2000; The Non-competitive Antagonists 2-Methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid Ethyl Ester Interact with Overlapping Binding Pockets in the Transmembrane Region of Group-I Metabotropic Glutamate Receptors. J Biol Chem. 275: 33750- 33758
- Parmentier ML , Galvez T , Acher F , Peyre B , Pellicciari R , Grau Y , Bockaert J , Pin JP 2000; Conservation of the ligand recognition site of metabotropic glutamate receptors during evolution. Neuropharmacology. 39: 1119- 31
- Parmentier ML , Joly C , Restituito S , Bockaert J , Grau Y , Pin J-P 1998; The G-protein coupling profile of metabotropic glutamate receptors, as determined with exogenous G-proteins, is independent of their ligand recognition domain. Mol Pharmacol. 53: 778- 86
- Pin JP , De Colle C , Bessis AS , Acher F 1999; New perspectives for the development of selective metabotropic glutamate receptor ligands. Eur J Pharmacol. 375: 277- 94
- Prezeau L , Carrette J , Helpap B , Curry K , Pin J-P , Bockaert J 1994; Pharmacological characterization of metabotropic Glutamate receptors in several types of brain cells in primary cultures. Mol Pharmacol. 45: 570- 577
- Prezeau L , Gomeza J , Ahern S , Mary S , Galvez T , Bockaert J , Pin J-P 1996; Changes of the C-terminal domain of mGluR1 by alternative splicing generate receptors with different agonist independent activity. Mol Pharmacol. 49: 422- 429
- Samama P , Cotecchia S , Costa T , Lefkowitz RJ 1993; A mutation-induced activated state of the β_2-adrenergic receptor. J Biol Chem. 268: 4625- 4636
- Schoepp DD , Jane DE , Monn JA 1999; Pharmacological agents acting at subtypes of metabotropic glutamate receptors. Neuropharmacology. 38: 1431- 76
- Smith PK , Krohn RI , Hermanson GT , Mallia AK , Gartner FH , Provenzano MD , Fujimoto EK , Goeke NM , Olson BJ , Klenk DC 1985; Measurement of protein using bicinchoninic acid. Anal Biochem. 150: 76- 85

Fig. 1

Chemical structure of BAY36-7620 ((3aS,6aS)-6a-Naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[c]furan-1-on), MPEP (2-Methyl-6-6(phenylethynyl)pyridine), CPCCOEt (7-Hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester) and L-glutamate.

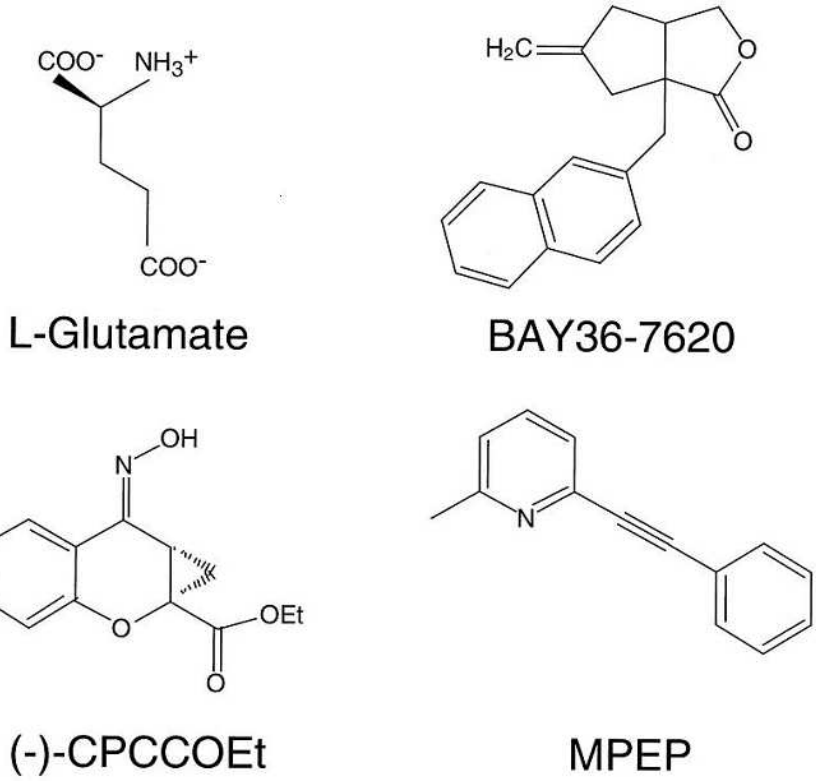


Fig. 2

BAY36-7620 alone has no effect on mGlu1 or mGlu5 receptors, however inhibits Glu-induced activation of mGlu1 but not mGlu5**. A:** BAY36-7620 is selective for mGlu1a over mGlu5a. IP accumulation was measured in HEK 293 cells transiently expressing mGlu1a, or 5a which were incubated with BAY36-7620 (10 μM), or Glu 1μM and 3μM respectively, in the presence of 0.1 & 10 μM BAY36-7620. The data are expressed as the percentage of IP formation measured with a submaximal Glu concentration. Data represent the mean ± s.e.m. of 4-16 experiments performed in triplicate. **B:** BAY36-7620 inhibits mGlu1 activation in a concentration-dependent manner. IP production by mGlu1 receptors transiently expressed in HEK 293 cells when activated with 1 μM of Glu, in the presence of increasing concentrations of BAY36-7620. Data are expressed as the ratio of IP formation over the radioactivity present in the solubilized fraction of the membranes. A representative of an experiment performed seven times in triplicate is shown.

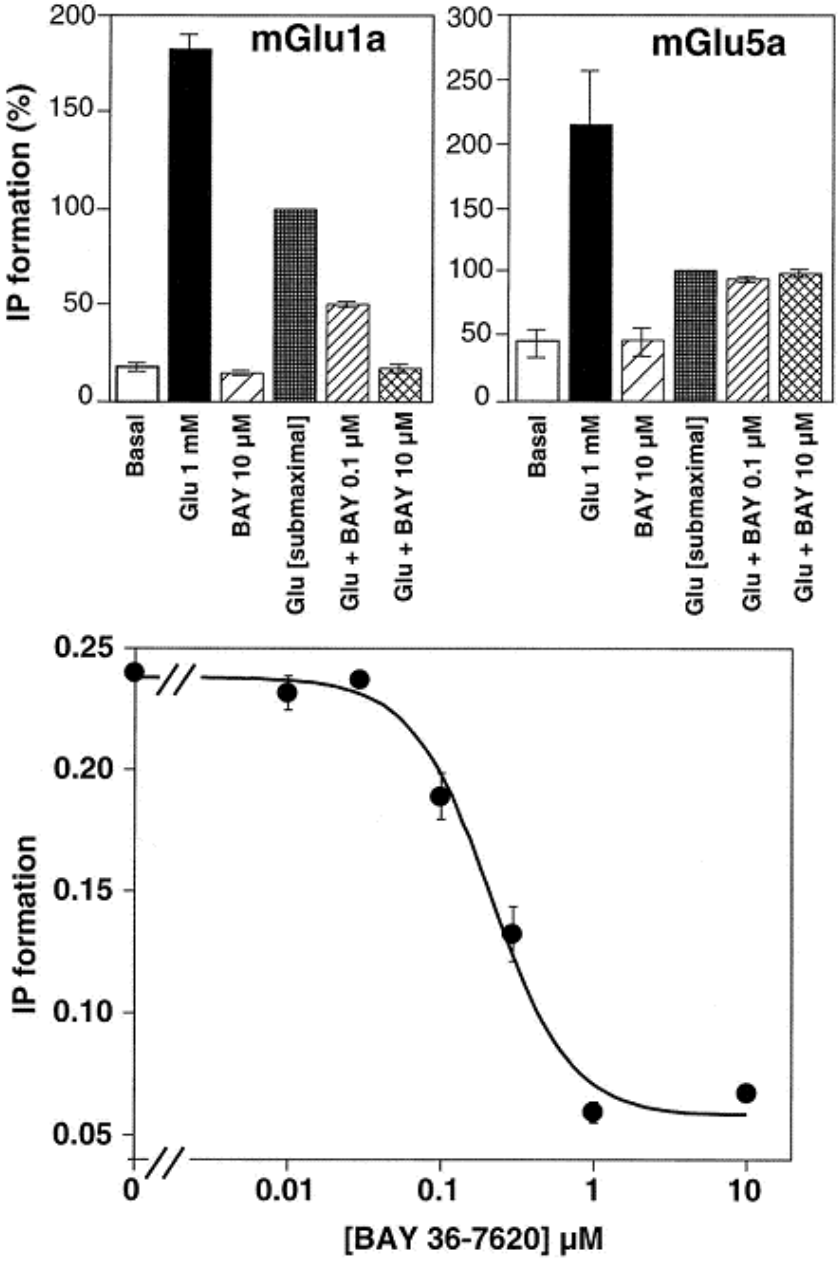


Fig. 3

BAY36-7620 has no effect on Group II & III mGlu receptors. IP accumulation was measured in HEK 293 cells transiently expressing mGlu2, 3, 4a, 6, 7a or 8a incubated with saturating concentrations of Glu [1 mM for mGlu1, 2, 3, , 5, 6 & 8 and 10 mM for mGlu7], with BAY36-7620 (10 μM), or with submaximal concentrations of Glu [20μM, 3μM, 30μM, 40μM, 5mM & 20μM respectively], in the presence and absence of 10 μM BAY36-7620. The data are expressed as the percentage of IP formation measured with a submaximal Glu concentration.. Data represent the mean ± s.e.m. of 2-3 experiments performed in triplicate.

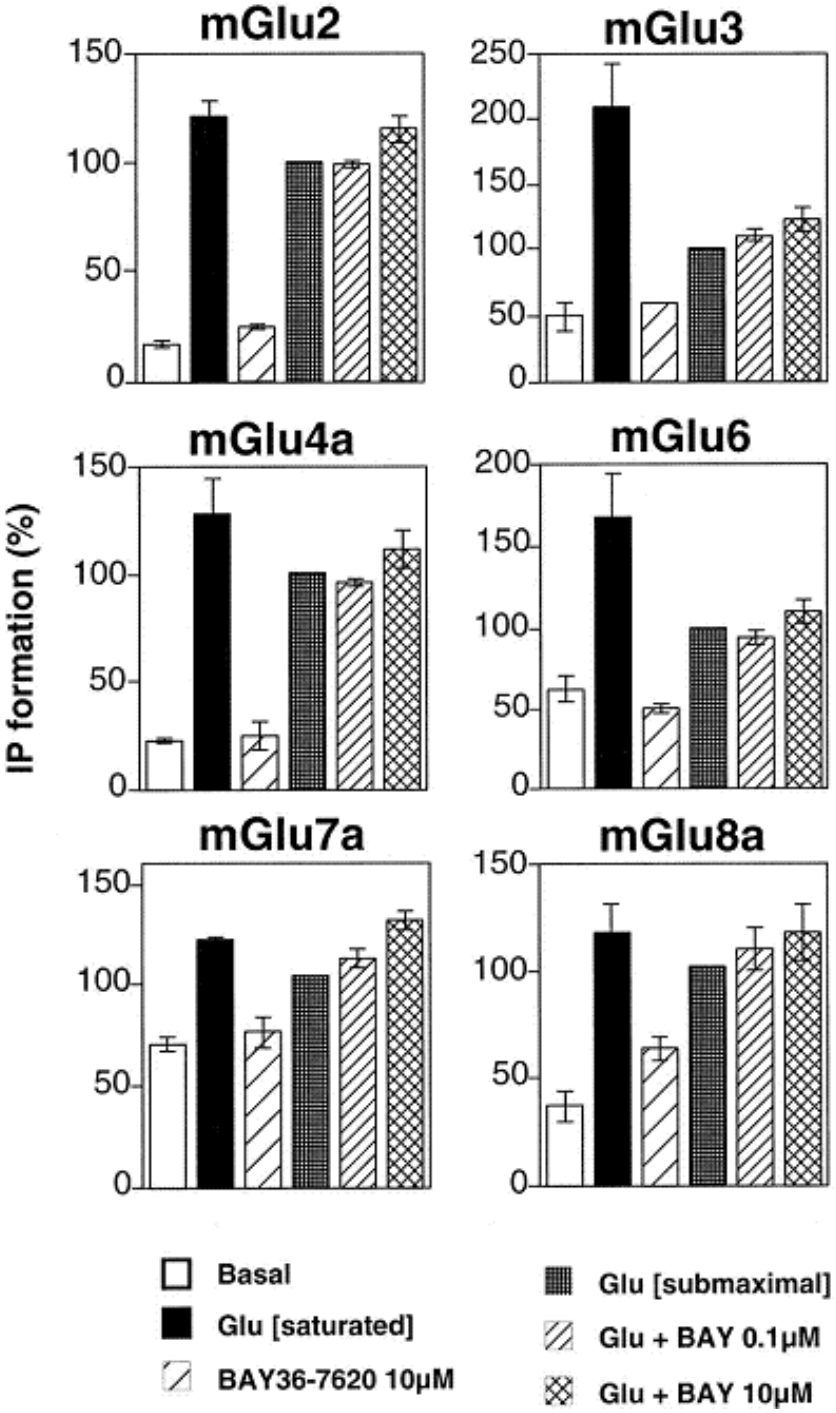


Fig. 4

BAY36-7620 is an inverse agonist at mGlu1a**. A:** Constitutive activity in HEK293 cells over-expressing mGlu1a is significantly inhibited by 10μM BAY36-7620 alone and reduces Glu-stimulated IP accumulation significantly below basal levels. Control cells were mock transfected with carrier protein pRK6 alone, **B:** BAY36-7620 (10μM) significantly inhibited basal and Glu-induced IP stimulation, to a level significantly lower than basal in HEK293 cells expressing mGlu1 and Gαq, whereas MCPG (3mM) whilst significantly attenuating the Glu-stimulated response did not inhibit constitutive activity in HEK293 cells expressing mGlu1a and Gαq. Control cells were mock transfected with carrier protein pRK6 and Gαq, **C:** Inhibition of constitutive activity by BAY36-7620 (0.01-100μM) in HEK293 cells expressing mGlu1a and Gαq, is concentration-dependent. Data are expressed as the ratio of IP formation over the radioactivity present in the solubilized fraction of the membranes and represent the mean ± s.e.m. of 2-6 experiments performed in triplicate. One-way ANOVA followed by post-hoc students T-tests were performed – for clarity, only pertinent differences are shown; * = significantly different from basal levels, † = significantly different from Glu 1μM, α =0.05.

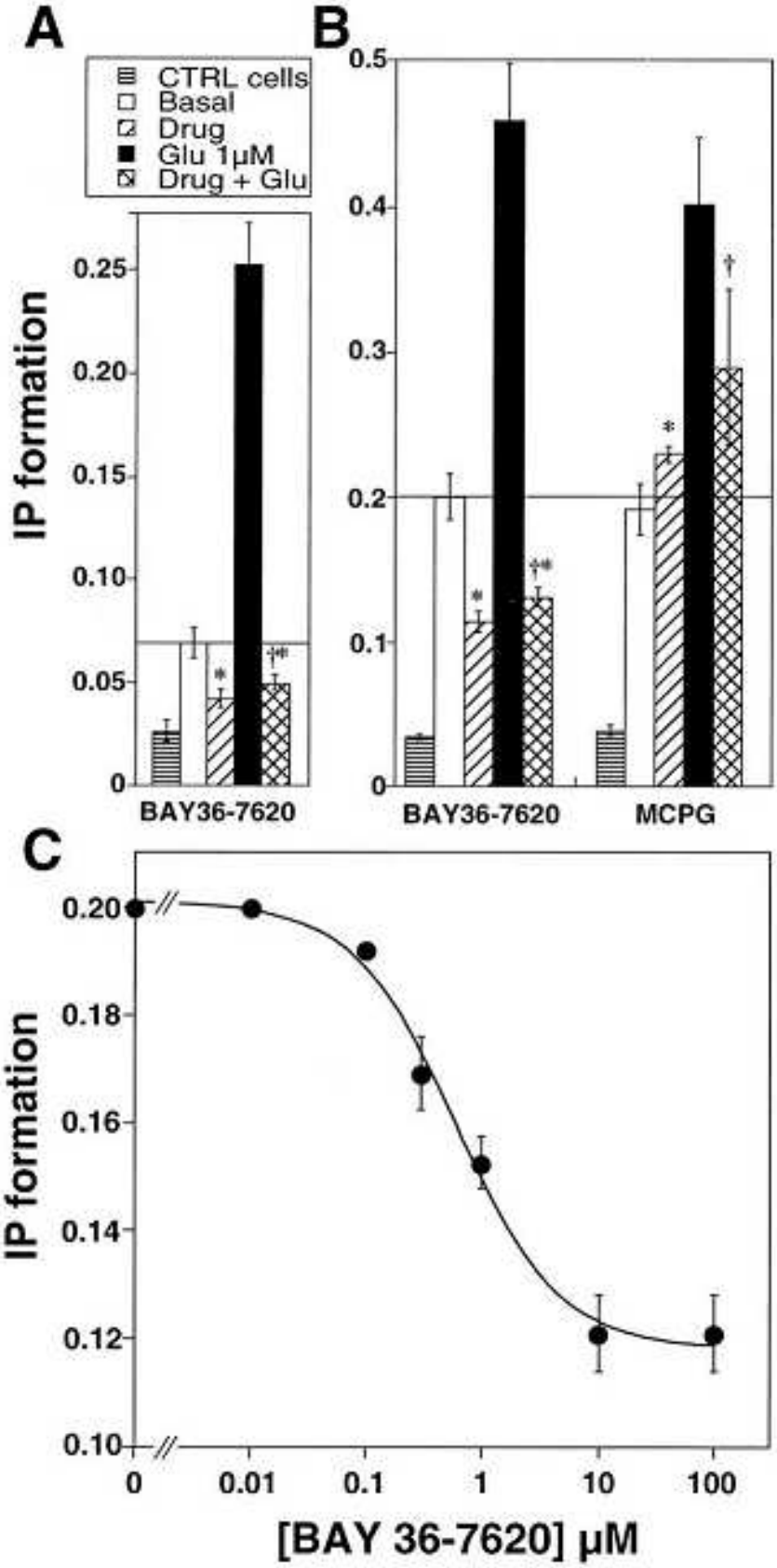

A
B
C
CTRL cells
Basal
Drug
Glu 1μM
Drug + Glu
IP formation
0.25
0.20
0.15
0.10
0.05
0
BAY36-7620
0.5
0.4
0.3
0.2
0.1
0
BAY36-7620
MCPG
IP formation
0.20
0.18
0.16
0.14
0.12
0.10
0
0.01
0.1
1
10
100
[BAY 36-7620] μM

Fig. 5

Schild analysis of the effect of BAY36-7620 on Glu activation of mGlu1 reveals that BAY36-7620 is a non-competitive antagonist of mGlu1. Full Glu concentration-responses were performed in the absence or presence of BAY36-7620 0.1; 1, & 10 μM on mGlu1a transiently expressed in HEK 293 cells. Data are expressed as the ratio of IP formation over the radioactivity present in the solubilized fraction of the membranes and a representative of an experiment performed three times in triplicate is shown.

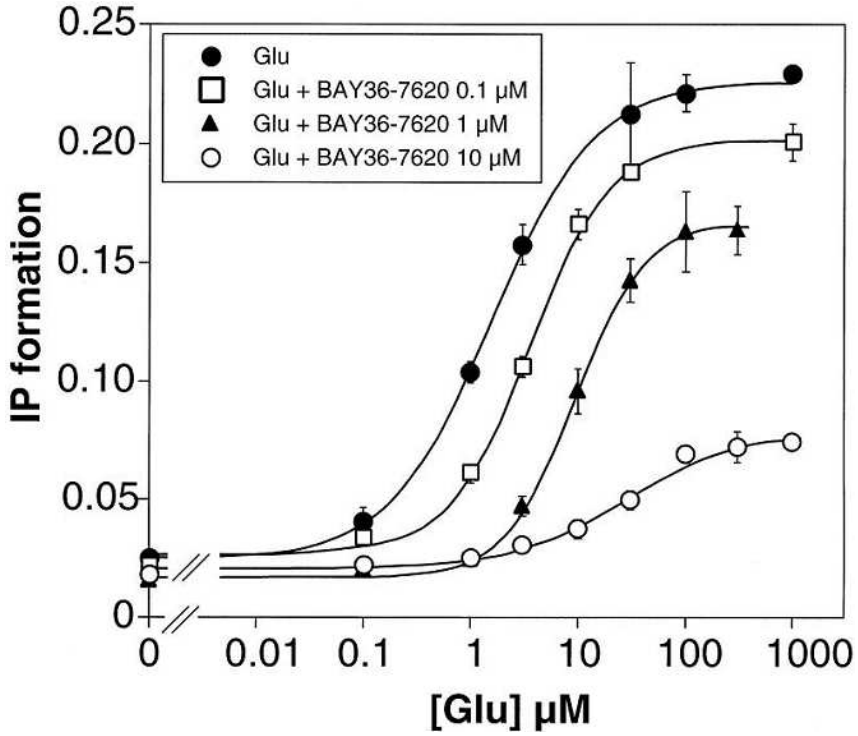


Fig. 6

BAY36-7620 does not affect [^{3}H]QA binding. Binding of 600nM [^{3}H]QA in HEK293 cells transiently transfected with mGlu1a was displaced by cold QA, but not by BAY36-7620. The presence of BAY36-7620 does affect the displacement of [^{3}H]QA by cold QA. The presented data are from a representative experiment among four experiments performed in triplicate.

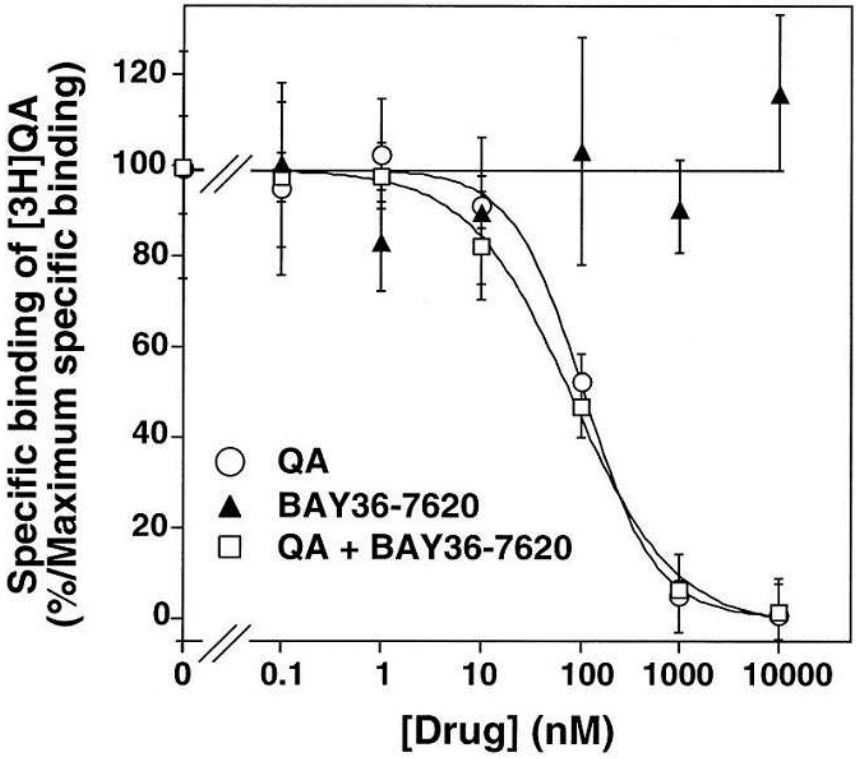


Fig. 7

BAY36-7620 interacts with the transmembrane region of mGlu1a**. A:** The mGlu1/DmGluRA chimeric receptor is not inhibited by BAY36-7620. The mGlu1/DmGluRA chimeric receptor and mGlu1a were expressed in HEK 293 cells and stimulated by 3μM and 1μM Glu respectively, in the presence and absence of 10μM BAY36-7620. Control cells were mock transfected with carrier protein pRK6 alone. Data are expressed as the ratio of IP formation over the radioactivity present in the solubilized fraction of the membranes and represent the mean ± s e.m. of 3 experiments performed in triplicate. **B:** BAY36-7620 inhibits Glu-induced IP production in HEK293 cells transfected with mGlu1a and the 2/1 mGlu chimera, but has no effect on the 1/2 mGlu chimera. Data are expressed as the ratio of IP formation over the radioactivity present in the solubilized fraction of the membranes and a representative of an experiment performed three times in triplicate is shown.

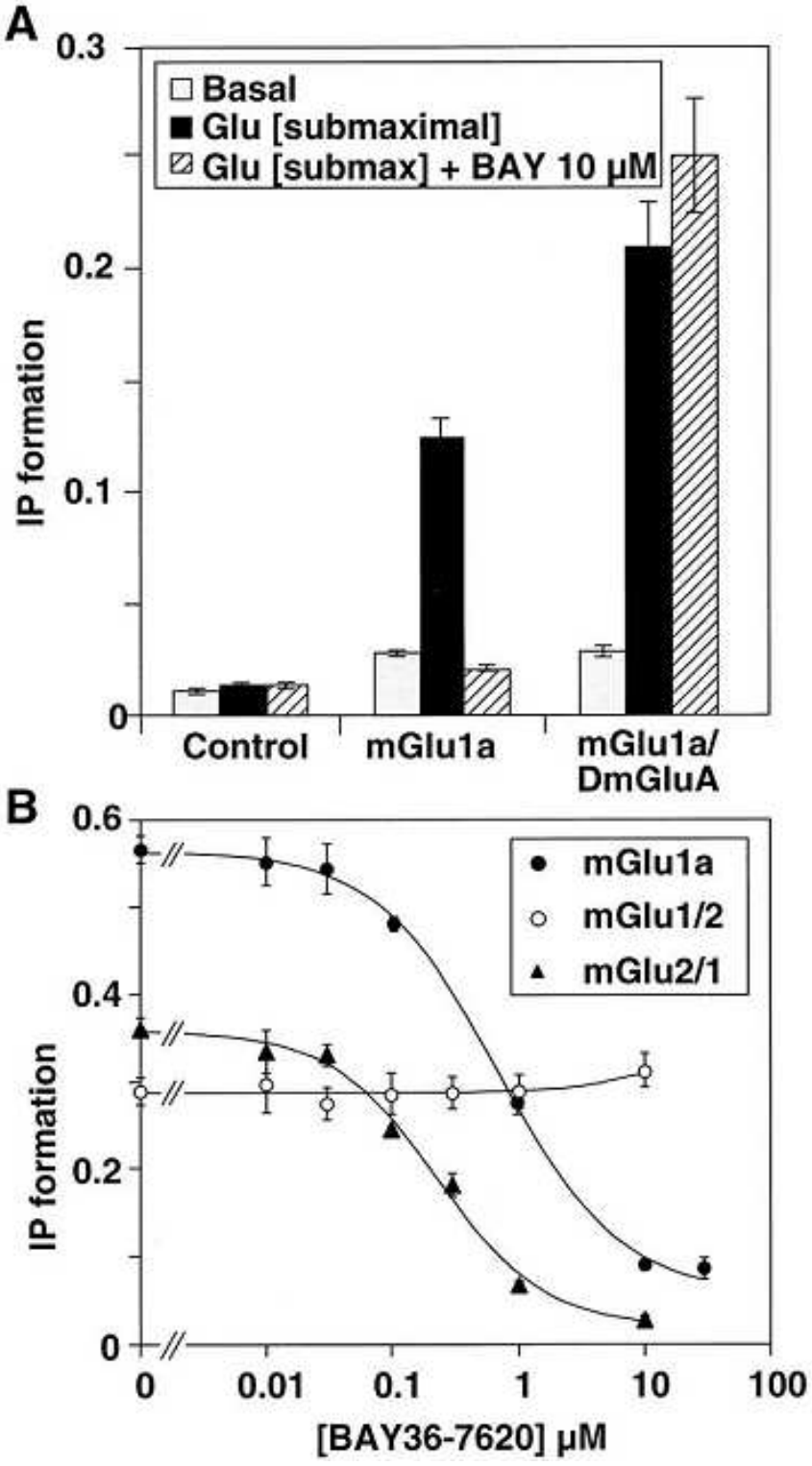


Fig. 8

The last four TM of mGlu1a are essential for the specificity of BAY36-7620. **A:** Schematic diagram of chimeric receptor constructs between mGlu1 and mGlu5, **B:** Chimeric receptors between mGlu1 & mGlu5 when expressed in HEK293 cells are responsive to Glu 3μM, but differ in their responsiveness to the antagonist BAY36-7620 (10μM). Data are expressed as the ratio of IP formation over the radioactivity present in the solubilized fraction of the membranes and represent the mean ± s. e. m. of 2-7 experiments performed in triplicate. **C:** The concentration-dependent antagonist effect of BAY36-7620 is similar in mGlu1a and chimeras C & F in which the last four TM and C terminal of mGlu1a remain common. Data are expressed as the ratio of IP formation over the radioactivity present in the solubilized fraction of the membranes and correspond to a representative experiment performed in triplicate. Identical data were obtained in two additional independent experiments. Students T-tests were performed on each chimera to ascertain the ability of BAY36-7620 to reduce the Glu-stimulated response. * = a significant attenuation, $\alpha = 0.05$.

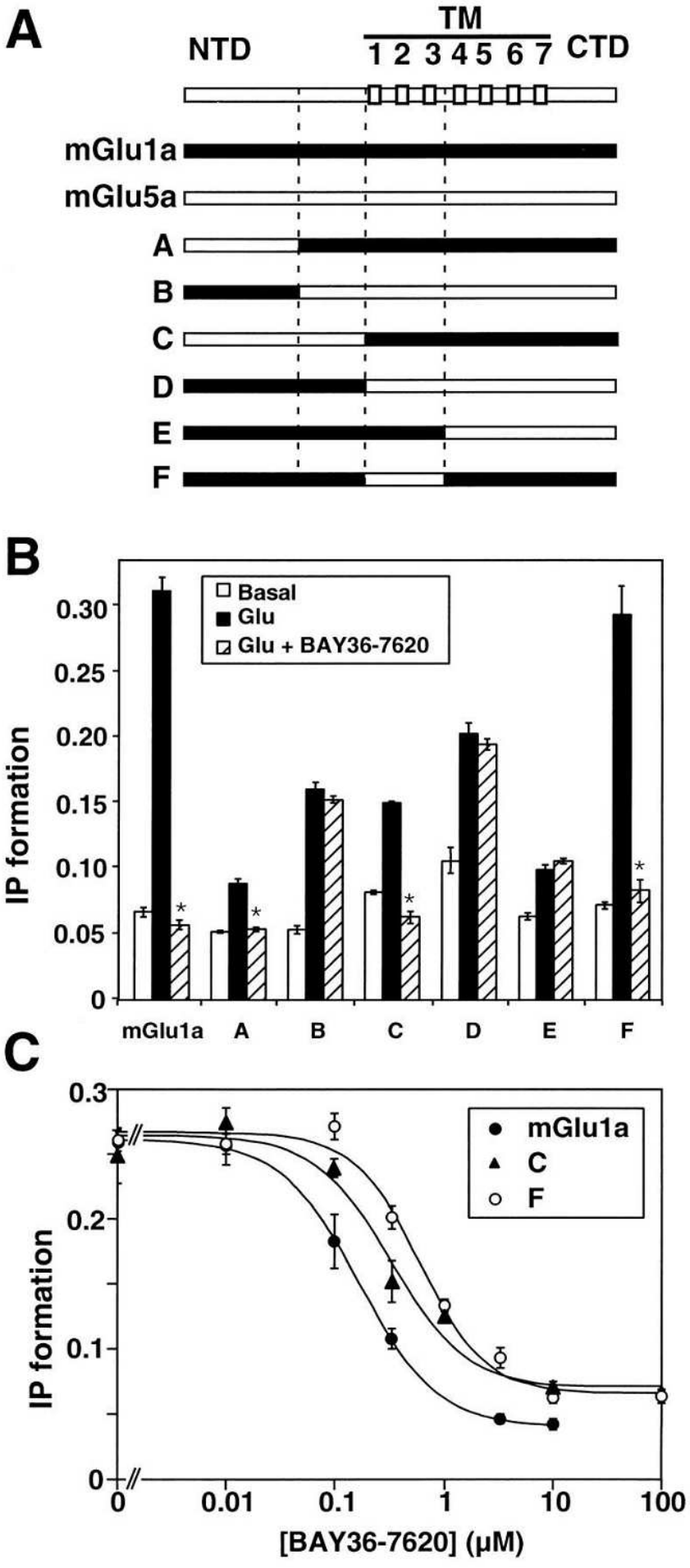

A
NTD
TM
1 2 3 4 5 6 7
CTD
mGlu1a
mGlu5a
A
B
C
D
E
F
B
Basal
Glu
Glu + BAY36-7620
IP formation
0.30
0.25
0.20
0.15
0.10
0.05
0
mGlu1a
A
B
C
D
E
F
C
mGlu1a
C
F
IP formation
0.3
0.2
0.1
0
0
0.01
0.1
1
10
100
[BAY36-7620] (μM)

TABLE 1

Potency (IC_{50}, μM) of BAY36-7620 in inhibiting the action of glutamate on the wild-type mGlu1 and various chimeric mGlu receptors. For each receptor, the origin of the ECD (from mGlu1, mGlu5) and the 7TM (from mGlu1, DmGluA, mGlu2, mGlu5 or mGlu1 and mGlu5 (1/5)) are indicated. Data are means ± s. e. m. of at least 3 independent experiments performed in triplicates. > 1 that no significant inhibition of the Glu-induced IP formation was detected with 100 μM BAY36-7620.

	mGlu1	**mGlu1/DA**	**mGlu1/2**	**mGlu2/1**	**mGlu1/5-D**	**mGlu5/1-C**	**mGlu5/1-F**
ECD	1	1	1	2	1	5	1
7TM	1	DA	2	1	5	1	5/1
$1C_{50}$ (μM)	0.16 ± 0.01	> 100	> 100	0.14 ± 0.05	> 100	0.24 ± 0.01	0.50 ± 0.06