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Pharmacology of (S)-homoquisqualic acid and (S)-2-amino-5-phosphonopentanoic acid [(S)-AP5] at cloned metabotropic glutamate receptors

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1 In this study we have determined the pharmacological profile of (**S**)-quisqualic acid, (**S**)-2-amino-4-phosphonobutyric acid ((**S**)-AP4) and their higher homologues (**S**)-homoquisqualic acid, (**S**)-2-amino-5-phosphonopentanoic acid ((**S**)-AP5), respectively, and (**R**)-AP5 at subtypes of metabotropic (**S**)-glutamic acid (mGlu) receptors expressed in Chinese hamster ovary cells.

2 (**S**)-Quisqualic acid was a potent $mGlu_1/mGlu_5$ agonist (EC_{50} values of 1.1 μM and 0.055 μM, respectively) showing no activity at $mGlu_2$ and weak agonism at $mGlu_4$ ($EC_{50} \sim 1000$ μM).

3 (**S**)-Homoquisqualic acid displayed competitive antagonism at $mGlu_1$ ($K_B = 184$ μM) and full agonism at $mGlu_5$ ($EC_{50} = 36$ μM) and $mGlu_2$ ($EC_{50} = 23$ μM), but was inactive at $mGlu_4$.

4 (**S**)-AP4 was a potent and selective $mGlu_4$ agonist ($EC_{50} = 0.91$ μM) being inactive at $mGlu_1$, $mGlu_2$ and $mGlu_5$ both as agonist and antagonist.

5 (**S**)-AP5 displayed very weak agonist activity at $mGlu_4$. At the $mGlu_2$ receptor subtype (**S**)-AP5 acted as a competitive antagonist ($K_B = 205$ μM), whereas the compound was inactive at $mGlu_1$ and $mGlu_5$. (**R**)-AP5 was inactive at all mGlu receptor subtypes tested both as agonist and antagonist.

6 These studies demonstrate that incorporation of an additional carbon atom into the backbone of (**S**)-glutamic acid and its analogues, to give the corresponding homologues, and replacement of the terminal carboxyl groups by isosteric acidic groups have profound effects on the pharmacological profiles at mGlu receptor subtypes. Furthermore, (**S**)-homoquisqualic acid has been shown to be a potentially useful tool for differentiating $mGlu_1$ and $mGlu_5$.

Keywords: Metabotropic glutamate receptors; mGluR; (**S**)-quisqualic acid; (**S**)-homoquisqualic acid; (**S**)-AP4 (L-AP4); (**S**)-AP5 (L-AP5); (**R**)-AP5 (D-AP5).

Introduction

(**S**)-Glutamic acid is the major excitatory amino acid neurotransmitter in the central nervous system and is involved in many important neural processes such as learning, memory, plasticity and neurotoxicity (Watkins *et al*., 1990; Bliss & Collingridge, 1993; Nakanishi & Masu, 1994). (**S**)-Glutamic acid receptors are divided into two distinct groups (Nakanishi & Masu, 1994; Hollmann & Heinemann, 1994). Ionotropic (**S**)-glutamic acid receptors are ligand activated cation channels which are further subdivided into three groups activated by *N*-methyl-D-aspartic acid (NMDA), (**S**)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid ((**S**)-AMPA) or kainic acid (Nakanishi & Masu, 1994; Hollmann & Heinemann, 1994; Krogsgaard-Larsen *et al*., 1996). Metabotropic (**S**)-glutamic acid (mGlu) receptors belong to the family of G-protein coupled receptors which regulate the intracellular content of second messengers (Nakanishi & Masu, 1994; Knöpfel *et al*., 1995; Pin & Duvoisin, 1995).

Based on molecular cloning, eight different subtypes of mGlu receptors have been identified (Nakanishi & Masu, 1994; Knöpfel *et al*., 1995; Pin & Duvoisin, 1995). On the basis of pharmacological characterization, sequence homology and second messenger pathways the receptors have been subdivided into three groups (Nakanishi & Masu, 1994; Knöpfel *et al*., 1995; Pin & Duvoisin, 1995): $mGlu_1$ and $mGlu_5$ form group I, which are coupled to phosphoinositide (PI) hydrolysis and are selectively activated by (**S**)-quisqualic acid (Aramori & Nakanishi, 1992; Abe *et al*., 1992); $mGlu_2$ and $mGlu_3$ form group II, which are coupled to inhibition of adenylate cyclase and are selectively activated by (2**S**,2′**R**,3′**R**)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG IV) and (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate (LY354740) (Hayashi *et al*., 1993; Schoepp *et al*., 1997); and $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$, which also are coupled to inhibition of adenylate cyclase, form group III and are selectively activated by (**S**)-2-amino-4-phosphonobutyric acid ((**S**)-AP4, L-AP4) (Nakanishi & Masu, 1994; Knöpfel *et al*., 1995; Pin & Duvoisin, 1995).

In recent studies, we have demonstrated pronounced changes in pharmacological profiles at mGlu receptors of analogues with varying backbone chain lengths (Bräuner-Osborne *et al*., 1996b; Krogsgaard-Larsen *et al*., 1996). Thus, (**S**)-2-aminoadipic acid, the higher homologue of the non-selective endogenous agonist (**S**)-glutamic acid (Figure 1), is capable of activating $mGlu_2$ and $mGlu_6$ but is inactive at $mGlu_1$ (Bräuner-Osborne *et al*., 1996b). Furthermore, (**S**)-2-amino-4-(3-hydroxy-5-methylisoxazol-4-yl)butyric acid ((**S**)-homo-AMPA), the higher analogue of the selective AMPA receptor agonist (**S**)-AMPA (Figure 1), is a specific $mGlu_6$ receptor and is inactive at AMPA receptors (Bräuner-Osborne *et al*., 1996b; Ahmadian *et al*., 1997). These results have led us to investigate the structure-activity relationships of the higher homologues of already known excitatory amino acid agonists. As already mentioned (**S**)-quisqualic acid is a potent and selective agonist at group I mGlu receptors. The higher homologue (**S**)-homoquisqualic acid has been shown to stimulate PI hydrolysis in rat brain cortex (Porter *et al*., 1992) and rat hippocampus (Littman *et al*., 1995), although with lower potency than the parent compound, (**S**)-quisqualic

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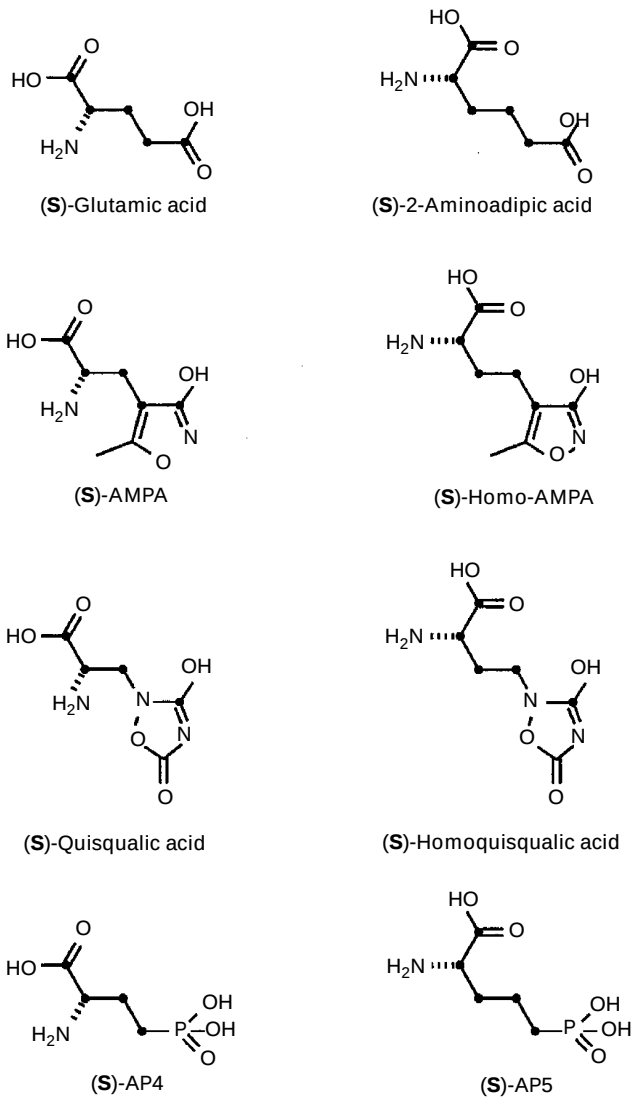

Figure 1 Structures of some excitatory amino acids and their higher homologues.

acid. (S)-2-Amino-5-phosphonopentanoic acid ((S)-AP5, L-AP5), the higher homologue of the selective group III agonist, (S)-AP4, has been found to be a $mGlu_{4a}$ receptor agonist although with lower potency than the parent compound (Johansen *et al*., 1995). Finally, the stereoisomer of (S)-AP5, (R)-AP5 (D-AP5) was included into this study as it has an antagonistic effect at excitatory amino acid receptors of the NMDA subtype (Evans *et al*., 1982). These previous studies indicated interactions of the higher amino acid homologues with mGlu receptors. However, in order to elucidate further their pharmacological profile, we studied the effects of these compounds at cloned mGlu receptors representative of all three groups (I – III).

Methods

Cell culture

The Chinese hamster ovary (CHO) cell lines expressing $mGlu_{1\alpha}$, $mGlu_2$, $mGlu_{4a}$ and $mGlu_{5a}$ have been described previously (Aramori & Nakanishi, 1992; Tanabe *et al*., 1992, 1993; Abe *et al*., 1992). The cell cultures were maintained at 37°C in a humidified 5% CO_2 incubator in Dubecco's modified Eagle medium (DMEM) with a reduced level of glutamine (2 mM) supplemented with 1% proline, penicillin (100 u ml^{-1}), streptomycin (100 mg ml^{-1}) and 10% dialysed foetal calf serum (all from GIBCO, Paisley, Scotland). For PI assays, 1.8 M cells were divided into the wells of 48 well plates two days before assay. Twenty four hours before the PI assay media were removed and exchanged with DMEM containing [^{3}H]-inositol (2 μCi ml^{-1}). For adenosine 3′:5′-cyclic monophoshate (cyclic AMP) assays, 1 M cells were divided into the wells of 96 well plates 2 days before assay.

Measurement of PI hydrolysis and cyclic AMP formation

PI assays were performed essentially as described previously (Hayashi *et al*., 1992, 1994). For agonist assay, the cells were incubated with ligand dissolved in phosphate buffered saline (PBS) supplemented with 10 mM LiCl for 20 min and agonist activity was determined by measurement of the level of ^{3}H-labelled mono-, bis- and tris-inositol phosphates by ion-exchange chromatography. For antagonist assay, the cells were preincubated with the ligand dissolved in PBS-LiCl for 20 min before incubation with ligand and 30 μM (S)-glutamic acid for 20 min. The antagonist activity was then determined as the inhibitory effect on the (S)-glutamic acid-mediated response. The assay of cyclic AMP formation was slightly modified compared to the previously described method (Hayashi *et al*., 1992, 1994). Briefly, the cells were incubated for 10 min in PBS containing the ligand, 10 μM forskolin and 1 mM isobutyl methylxanthine (IBMX, both Sigma Chemicals). The agonist activity was then determined as the inhibitory effect of forskolin-induced cyclic AMP formation. For antagonist assay the cells were preincubated with ligand dissolved in PBS containing 1 mM IBMX for 20 min before a 10 min incubation in PBS containing the ligand, 30 μM (S)-glutamic acid, 10 μM forskolin and 1 mM IBMX. Cyclic AMP levels were determined by the use of a scintillation proximity assay according to the manufacturer's protocol (Amersham).

Materials

(S)-Glutamic acid was purchased from Sigma Chemicals (St. Louis, MO). (S)-Quisqualic acid, (S)-homoquisqualic acid, (S)-AP4, (S)-AP5 and (R)-AP5 were purchased from Tocris Cookson (Bristol, U.K.).

Data analysis

All experiments were performed in triplicate and the results are given as mean ± s.e.mean of at least two independent experiments. Antagonist potency was calculated from the Gaddum equation $K_B = [B]/(DR-1)$ (Lazareno & Birdsall, 1993), where the dose-ratio (DR) is the ratio of the EC_{50} values of (S)-glutamic acid in the presence or absence of a fixed antagonist concentration, [B]. Statistical significance was checked by Student's *t* test for unpaired data.

Results

Initially, (S)-quisqualic acid, (S)-homoquisqualic acid, (S)-AP4, (S)-AP5 and (R)-AP5 were tested in 1 mM concentration at CHO cell lines expressing $mGlu_{1\alpha}$, $mGlu_2$ and $mGlu_{4a}$ receptors representing group I, II and III, respectively. In agreement with previous studies (Aramori & Nakanishi, 1992; Tanabe *et al*., 1992, 1993), (S)-quisqualic acid selectively

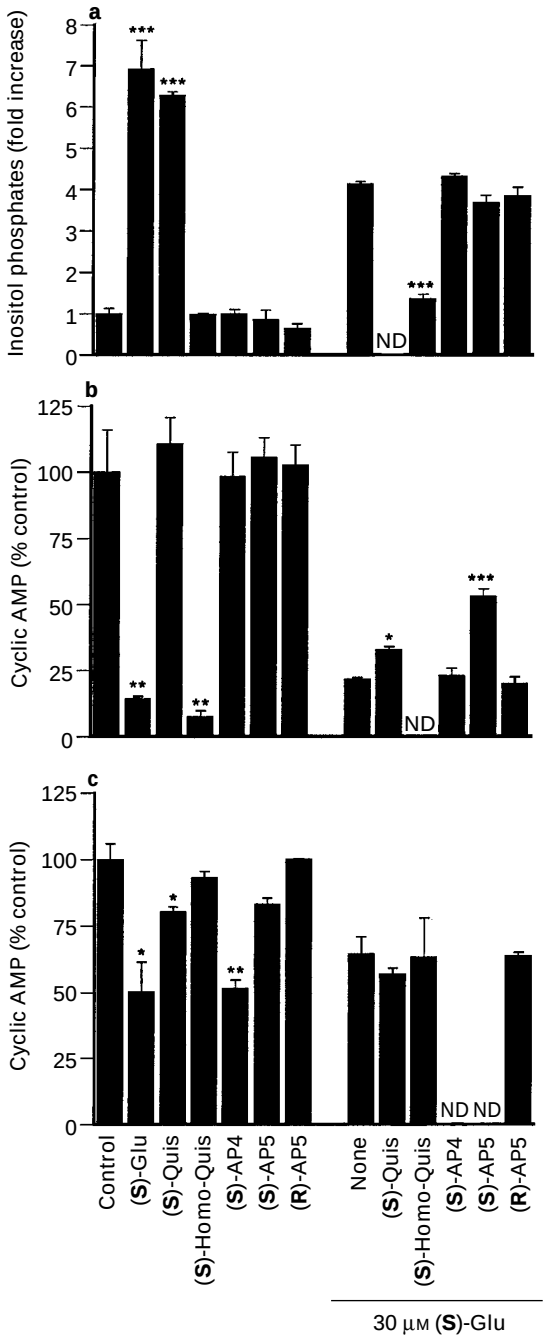

Figure 2 Agonist and antagonist activities at CHO cells expressing $mGlu_{1\alpha}$, $mGlu_2$ or $mGlu_{4a}$ receptors. $mGlu_{1\alpha}$-expressing (a) cells were incubated with test compound at a concentration of 1 mM for 20 min in the absence (agonist assay) or presence (antagonist assay) of 30 μM (S)-glutamic acid. In antagonist assays, cells were preincubated with test compound for 20 min. Total IP formation was determined by ion-exchange assay and fold increase in IP level calculated compared to control cells (incubated in buffer only, 217 $\pm$ 11 d.p.m.). $mGlu_2$-expressing (b) and $mGlu_{4a}$-expressing (c) cells were incubated with test compound (1 mM) for 10 min in the presence of 10 μM forskolin and in the absence (agonist assay) or presence (antagonist assay) of 30 μM (S)-glutamic acid. In antagonist assays, cells were preincubated with test compound for 20 min. Cyclic AMP levels in forskolin-treated cells were 26 $\pm$ 3 pmol per well ($mGlu_2$-expressing cells) and 2.6 $\pm$ 0.2 pmol per well ($mGlu_{4a}$-expressing cells). Forskolin treatment increased cyclic AMP levels by 38 and 20 fold, respectively. Cyclic AMP levels were measured by a scintillation proximity assay (Amersham) and were set to 100% in control cells (forskolin treated cells incubated in buffer only). Data are the means ($\pm$ s.d.) of representative experiments performed in triplicate. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (S)-Glu, (S)-glutamic acid; (S)-Quis, (S)-quisqualic acid; (S)-Homo-Quis, (S)-homoquisqualic acid.

activated $mGlu_{1\alpha}$ being inactive at $mGlu_2$ and showing very weak agonist activity at $mGlu_{4a}$ (Figure 2). As shown in Figure 3a and Table 1, (S)-quisqualic acid was about 10 fold more potent than (S)-glutamic acid at $mGlu_{1\alpha}$, which is also in agreement with previous findings (Aramori & Nakanishi,

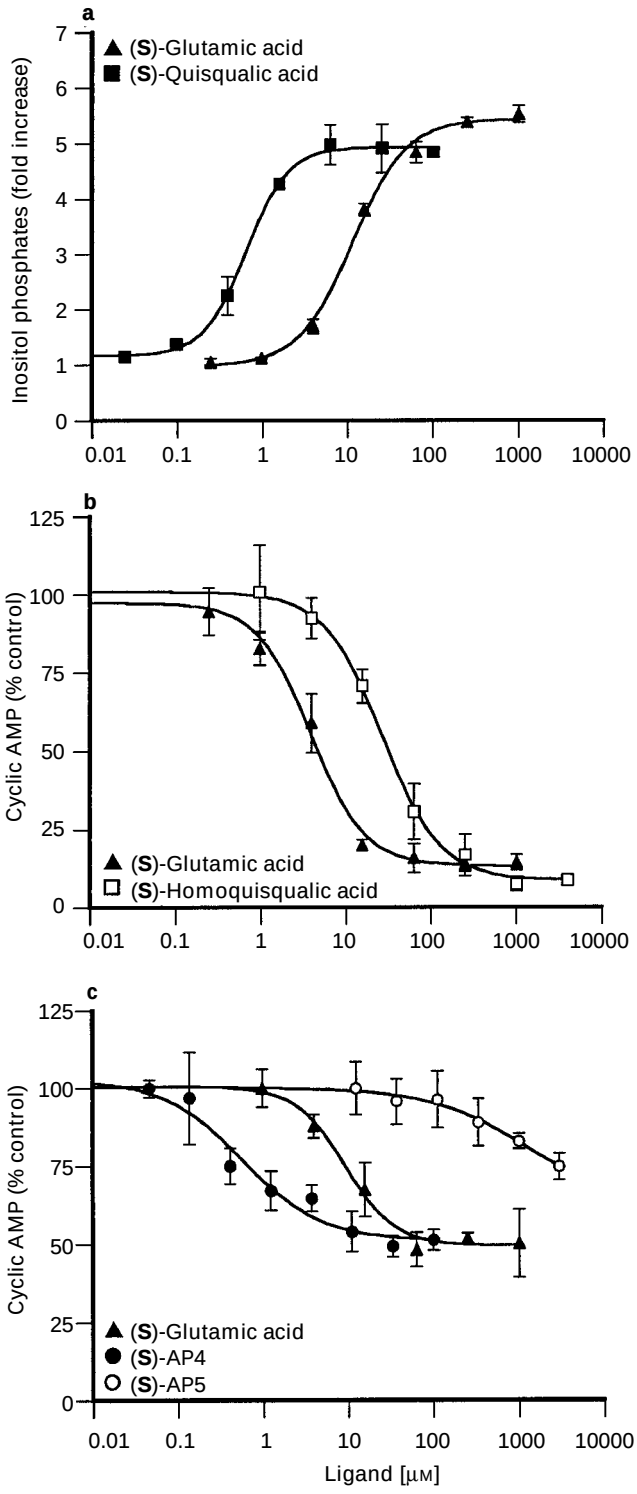

Figure 3 Dose-response curves of test compounds with agonist activities at CHO cells expressing $mGlu_{1\alpha}$ (a), $mGlu_2$ (b) or $mGlu_{4a}$ (c). Basal level of total inositol phosphates was 222 $\pm$ 32 d.p.m. at $mGlu_{1\alpha}$-expressing cells. Cyclic AMP levels in forskolin-treated cells were 2.5 $\pm$ 0.1 pmol per well ($mGlu_2$-expressing cells) and 4.0 $\pm$ 0.2 pmol per well ($mGlu_{4a}$-expressing cells). Forskolin treatment increased cyclic AMP levels by 29 and 30 fold in $mGlu_2$- and $mGlu_{4a}$-expressing cells, respectively. For other details, see Figure 2.

1992). (**S**)-Homoquisqualic acid, on the other hand, was inactive when tested as an agonist at $mGlu_{1\alpha}$ whereas it displayed antagonism at this receptor subtype (Figure 2a). When tested in further detail by rightward shifting of (**S**)-glutamic acid dose-response curves (Figure 4a), a K_B value of 184 μM was determined (Table 1). (**S**)-Homoquisqualic acid caused a parallel rightward shift indicating competitive antagonism (Figure 4a). As shown in Figure 3b and Table 1, (**S**)-homoquisqualic acid also displayed agonism at $mGlu_2$, being some 5 fold less potent than (**S**)-glutamic acid, while being inactive at $mGlu_{4a}$ (Figure 2c). In agreement with previous studies (Aramori & Nakanishi, 1992; Tanabe *et al*., 1992, 1993), (**S**)-AP4 selectively activated $mGlu_{4a}$, being inactive at $mGlu_{1\alpha}$ and $mGlu_2$ (Figure 2). Also in agreement with these studies, (**S**)-AP4 was approximately 10 fold more potent at this receptor subtype than (**S**)-glutamic acid (Figure 3c and Table 1). As shown in Figure 3c and Table 1, (**S**)-AP5 displayed about 1000 fold less agonist activity at $mGlu_{4a}$ than (**S**)-AP4, in agreement with a 200 fold loss of activity found previously (Johansen *et al*., 1995). However, as shown in Figure 2b, (**S**)-AP5 was able to antagonize a (**S**)-glutamic acid-mediated response at the $mGlu_2$ receptor subtype. When studied in further detail by right-shifting of (**S**)-glutamic acid dose-response curves, as shown in Figure 4b, a shift was induced corresponding to a K_B value of 205 μM (Table 1). (**S**)-AP5 displayed no activity at the $mGlu_{1\alpha}$ receptor subtype (Figure 2a and Table 1). Finally, (**R**)-AP5 was found to be inactive at all three receptor subtypes both when tested for agonist and antagonist effects (Figure 2 and Table 1).

As mentioned previously, (**S**)-homoquisqualic acid has been shown to stimulate PI-hydrolysis in rat brain cortex and rat hippocampus, indicative of group I agonism (Littman *et al*., 1995; Porter *et al*., 1992). This apparent disagreement with our results showing $mGlu_{1\alpha}$ antagonism prompted us to test the compounds at $mGlu_{5a}$, which is the second member of group I. As shown in Figure 5 and Table 1, (**S**)-quisqualic acid, (**S**)-glutamic acid and (**S**)-homoquisqualic acid all displayed full agonism at this receptor subtype (maximal responses not statistically different, $P > 0.05$) with EC_{50} values of 0.055 μM, 4.4 μM and 36 μM, respectively, whereas (**S**)-AP4, (**S**)-AP5 and (**R**)-AP5 were inactive both as agonists and antagonists.

Discussion

In this study we have tested (**S**)-homoquisqualic acid, (**S**)-AP5, which are the higher homologues of (**S**)-quisqualic acid and (**S**)-AP4, respectively, and (**R**)-AP5 at the cloned mGlu receptors. These studies have revealed rather complex molecular pharmacological profiles of these homologous compounds. Thus, whereas (**S**)-quisqualic acid is a potent and selective group I ($mGlu_1$, $mGlu_5$) agonist, (**S**)-homoquisqualic acid is an antagonist at $mGlu_{1\alpha}$. These results are in apparent disagreement with previously published observations, in which (**S**)-homoquisqualic acid was shown to stimulate PI hydrolysis in rat cortex and hippocampus although with weaker potency than (**S**)-quisqualic acid (Littman *et al*., 1995; Porter *et al*.,

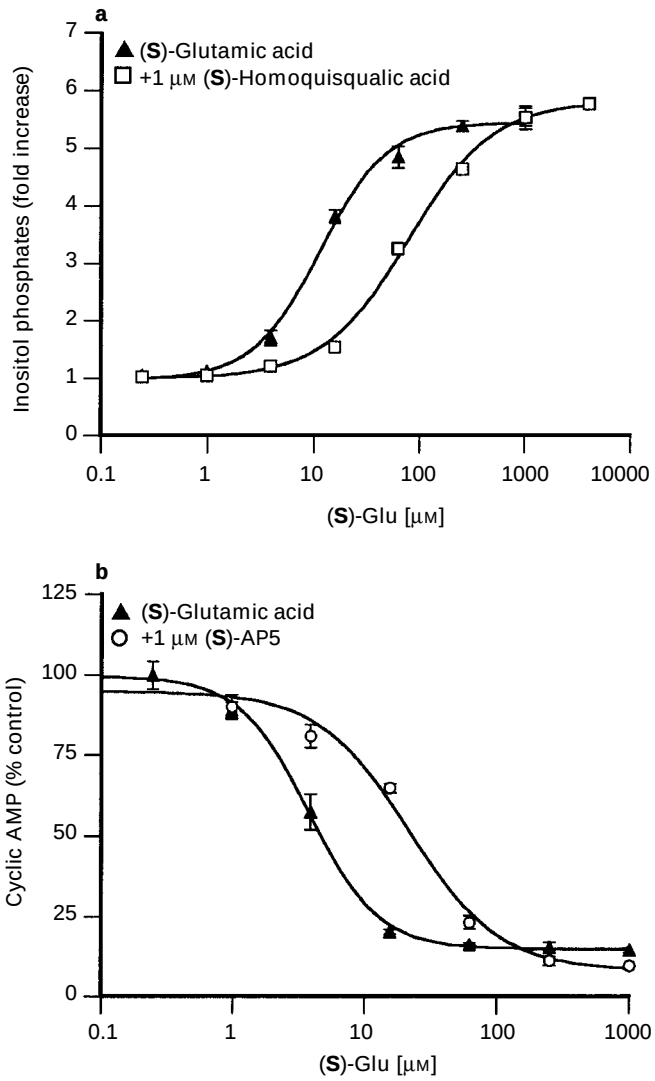

Figure 4 (a) Dose-response curves of (**S**)-glutamic acid in the absence or presence of 1 mM (**S**)-homoquisqualic acid at $mGlu_{1\alpha}$-expressing CHO cells. (b) Dose-response curves of (**S**)-glutamic acid in the absence or presence of 1 mM (**S**)-AP5 at $mGlu_2$-expressing CHO cells. For IP and cyclic AMP levels, see Figure 3. For other details, see Figure 2.

Table 1 Agonist and antagonist potencies of excitatory amino acid analogues and homologues tested on CHO cells expressing $mGlu_{1\alpha}$, $mGlu_2$, $mGlu_{4a}$ or $mGlu_{5a}$ receptors

	EC_{50} or K_B values (μM)			
	$mGlu_{1\alpha}$	*$mGlu_2$*	*$mGlu_{4a}$*	*$mGlu_{5a}$*
(**S**)-Glutamic acid	10±2	4.0±0.5	12±2	4.4±0.8
(**S**)-2-Aminoadipic acid[a]	>1000	35±1	>3000	ND
(**S**)-Quisqualic acid	1.1±0.1	>1000	~1000	0.055±0.016
(**S**)-Homoquisqualic acid	**184±19**	23±4	>1000	36±7
(**S**)-AP4	>1000	>1000	0.91±0.50	>1000
(**S**)-AP5	>1000	**205±31**	~1000	>1000
(**R**)-AP5	>1000	>1000	>1000	>1000

Data in plain and bold text are agonist and antagonist potencies, respectively, and are means±s.e.mean of at least three independent experiments. [a]From Bräuner-Osborne *et al*. (1996b).

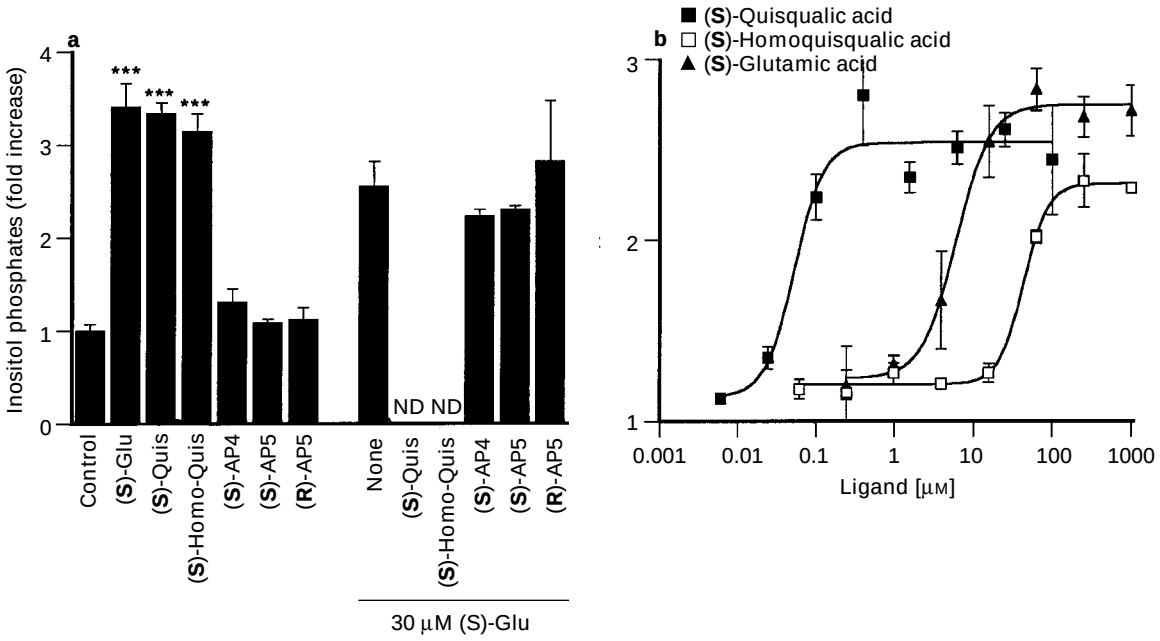

Figure 5 Agonist and antagonist activities at CHO cells expressing $mGlu_{5a}$ receptors. (a) $mGlu_{5a}$-expressing cells were incubated with test compound at a concentration of 1 mM in the absence (agonist assay) or presence (antagonist assay) of 30 μM (**S**)-glutamic acid. (b) Dose-response curves for test compounds which displayed agonist activity at $mGlu_{5a}$. Basal level of total inositol phosphates was 189 ± 14 d.p.m. For other details, see Figure 2.

1992). This apparent disagreement prompted us to test the compounds at the $mGlu_{5a}$ receptor subtype, the second member of group I which stimulates PI hydrolysis. As seen in Figure 5b, (**S**)-quisqualic acid and (**S**)-homoquisqualic acid were both full agonists at this receptor subtype, showing potencies in agreement with those measured in rat cortex and hippocampus (Littman *et al*., 1995; Porter *et al*., 1992). These results indicate that the (**S**)-homoquisqualic acid-stimulated PI hydrolysis in rat cortex and hippocampus are mediated by $mGlu_{5a}$ and not by $mGlu_{1\alpha}$. This actually is in agreement with the expression pattern of these receptor subtypes. Thus, $mGlu_{1\alpha}$ is not expressed in rat cortex and only at low levels in rat hippocampus (Masu *et al*., 1991; Catania *et al*., 1994), whereas $mGlu_{5a}$ is highly expressed in both of these brain regions (Abe *et al*., 1992; Shigemoto *et al*., 1993). Pharmacological differences between $mGlu_{1\alpha}$ and $mGlu_{5a}$ have been obtained previously. Thus, both (**RS**)-1-aminoindane-1,5-dicarboxylic acid (Moroni *et al*., 1997), (**S**)-4-carboxyphenylglycine and (+)-α-methyl-4-carboxyphenylglycine (Brabet *et al*., 1995) are more potent antagonists at $mGlu_{1\alpha}$ than at $mGlu_{5a}$. Furthermore, like (**S**)-homoquisqualic acid, (**S**)-4-carboxy-3-hydroxyphenylglycine is an antagonist at $mGlu_{1\alpha}$ and an agonist at $mGlu_{5a}$. Thus, like these compounds, (**S**)-homoquisqualic acid might be used to discriminate pharmacologically between $mGlu_{1\alpha}$ and $mGlu_{5a}$. It is interesting to note these pharmacological differences between $mGlu_{1\alpha}$ and $mGlu_{5a}$ which both belong to group I and share 87.1 % amino acid homology (including conserved substitutions, Abe *et al*., 1992). Most striking is the agonist/antagonist profiles of (**S**)-homoquisqualic acid and (**S**)-4-carboxy-3-hydroxyphenylglycine, allthough it should be cautioned that expressing levels of G-protein coupled receptors does influence intrinsic activity of ligands (Bräuner-Osborne *et al*., 1996a). Unfortunately, due to the lack of high-affinity radioligands for the group I mGlu receptors, the receptor levels of $mGlu_{1\alpha}$ and $mGlu_{5a}$ cannot be accurately determined. Therefore, the possibility that the agonist/antagonist profiles of compounds within group I, obtained by us and others, are caused by differences in receptor expression levels, cannot be ruled out. On the other hand, as little as one amino acid substitution has been shown to change classical antagonists to full agonists on the μ- and δ-opioid receptors (Claude *et al*., 1996). Thus it is plausible that the differences in amino acid sequence between $mGlu_{1\alpha}$ and $mGlu_{5a}$ are the cause of the pharmacological differences found by us and others.

Another interesting difference between (**S**)-quisqualic acid and (**S**)-homoquisqualic acid is their activity at $mGlu_2$. Whereas (**S**)-quisqualic acid is completely inactive at this receptor subtype, (**S**)-homoquisqualic acid is a rather potent agonist being approximately some 5 fold less potent than (**S**)-glutamic acid.

In agreement with a previous study, (**S**)-AP5 was a rather weak $mGlu_{4a}$ agonist compared to the potent and selective parent compound (**S**)-AP4 (Johansen *et al*., 1995). When tested on mGlu receptor subtypes from the two other subgroups (I and II), (**S**)-AP5 was found to be an antagonist at $mGlu_2$ (Figure 4b), showing that (**S**)-AP5 also possesses a dual agonist/antagonist profile. Similar agonist/antagonist profiles have also been demonstrated for carboxyphenylglycine derivatives, although these compounds are $mGlu_2$ agonists and $mGlu_{1\alpha}$ antagonists (Sekiyama *et al*., 1996).

It is interesting to note the impact on the pharmacological profile of these compounds of increasing the backbone chain length by one carbon. We have previously published similar observations for homologues of (**S**)-glutamic acid and (**S**)-AMPA (Bräuner-Osborne *et al*., 1996b; Ahmadian *et al*., 1997). Thus, whereas (**S**)-glutamic acid is a nonselective ligand at the mGlu receptors, the higher homologue, (**S**)-2-aminoadipic acid is selective for the $mGlu_2$ and $mGlu_6$ receptor subtypes (Table 1). Furthermore, (**S**)-homo-AMPA, the higher homologue of the selective AMPA agonist, (**S**)-AMPA, is a specific $mGlu_6$ receptor agonist (Bräuner-Osborne *et al*., 1996b; Ahmadian *et al*., 1997). Thus, based on the compounds investigated in this and previous studies, it can be concluded that no simple relationship between backbone chain length of (**S**)-glutamic

acid receptor ligands and pharmacological activity exists. These results point to the fact that backbone chain length and structure of the terminal acidic group are important parameters to consider when designing new selective mGlu receptor ligands.

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