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Characterization of an N-terminal secreted domain of the type-1 human metabotropic glutamate receptor produced by a mammalian cell line

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Abstract

A Chinese hamster ovary cell line has been established which secretes the N-terminal domain of human mGlu1 receptor. The secreted protein has been modified to contain a C-terminal hexa-histidine tag and can be purified by metal-chelate chromatography to yield a protein with an apparent molecular weight of 130 kDa. Following treatment with dithiothreitol the apparent molecular weight is reduced to 75 kDa showing that the protein is a disulphide-bonded dimer. N-terminal protein sequencing of both the reduced and unreduced forms of the protein yielded identical sequences, confirming that they were derived from the same protein, and identifying the site of signal-peptide cleavage of the receptor as residue 32 in the predicted amino acid sequence. Endoglycosidase treatment of the secreted and intracellular forms of the protein showed that the latter was present as an endoglycosidase H-sensitive dimer, indicating that dimerization is taking place in the endoplasmic reticulum. Characterization of the binding of [^{3}H]quisqualic acid showed that the protein was secreted at levels of up to 2.4 pmol/mL and the secreted protein has a K_d of 5.6 ± 1.8 nM compared with 10 ± 1 nM for baby hamster kidney (BHK)-mGlu1α receptor-expressing cell membranes. The secreted protein maintained a pharmacological profile similar to that of the native receptor and the binding of glutamate and quisqualate were unaffected by changes in Ca^{2+} concentration.

Keywords: G protein-coupled receptor, LY 367385, metabotropic glutamate receptor, [^{3}H]quisqualate, receptor dimerization, receptor glycosylation.

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The metabotropic glutamate (mGlu) receptors form a family of eight gene products that are G protein-coupled receptors activated by glutamate in the central nervous system (Tanabe *et al*. 1992). The mGlu receptors can be subdivided into three groups on the basis of their pharmacology, second messenger pathways and protein sequences (Pin and Duvoisin 1995; Knöpfel and Gasparini 1996; Conn and Pin 1997). The group-1 mGlu receptors are mGlu1 and mGlu5, which couple to the hydrolysis of inositol phospholipids and are predominantly located postsynaptically in the central nervous system. The second group consists of mGlu2 and mGlu3, which couple negatively to adenylyl cyclase and for which (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) is a potent and selective agonist. The third group contains mGlu4, mGlu6, mGlu7 and mGlu8, which also couple negatively to adenylyl cyclase, and have L-AP4 as a selective agonist (Pin and Duvoisin 1995; Knöpfel and Gasparini 1996; Conn and Pin 1997).

All members of the mGlu receptor family are presumed to have seven transmembrane domains, a large extracellular domain, and may be present as disulphide-bonded dimers in the plasma membrane (Romano *et al*. 1996). Several lines of evidence suggest that the extracellular N-terminal domain

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Abbreviations used: BHK, baby hamster kidney; CHO, Chinese hamster ovary; DCG-IV, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine; DTT, dithiothreitol; ECL, enhanced chemiluminescence; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; LY, 367385, (*S*)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid; mGlu, metabotropic glutamate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; SDS, sodium dodecylsulfate.

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contains the glutamate binding site. First, modelling studies suggest a homology between this domain of the mGlu receptors and bacterial periplasmic binding proteins, and site-directed mutagenesis based on this modelling altered the binding of agonist to the mGlu1 receptor (O'Hara *et al*. 1993). Second, chimeric proteins, in which the N-terminal domain of mGlu receptors from different groups was exchanged, caused the predicted alteration in the pharmacology of the responses of the mutated receptors, without affecting their second messenger responses (Takahashi *et al*. 1993; Tones *et al*. 1995). Finally, truncated forms of mGlu1α and mGlu4 receptors have been shown to be secreted from bacculovirus-infected insect cells, or transiently transfected HEK 293 cells, respectively, and to retain agonist binding activity (Okamoto *et al*. 1998; Han and Hampson 1999; Tsuji *et al*. 2000). Secreted forms of the N-terminal domain of the receptor therefore provide a useful resource for both the crystallization of this region of these receptors and for studying the interaction of ligands with the binding site, provided that the secreted proteins can be obtained in sufficient yield and purity. This has recently been shown to be the case with the successful structure determination of just such a secreted mGlu1 receptor domain both in a ligand-free and glutamate-bound form (Kunishima *et al*. 2000).

Because of differences in the glycosylation patterns of proteins in insect and mammalian cells, the latter might be envisaged as optimal for the production of appropriately glycosylated, secreted proteins. To overcome the difficulty of obtaining large quantities of protein from transiently transfected mammalian cells, a cell line permanently secreting the N-terminus of human mGlu1 receptor is needed, preferably with the secreted protein tagged to enable easy purification. Therefore, we have introduced a sequence of six histidine residues followed by a stop codon into the N-terminal domain of the human mGlu1 receptor, at a position just before the proposed first transmembrane domain. The resulting construct was stably transfected into Chinese hamster ovary cells, which secrete the N-terminal domain protein as a disulphide-bonded soluble protein dimer that can be readily purified on metal-chelate columns. Here we have characterized the secreted protein, and have shown that it maintains pharmacological specificity and exhibits a higher affinity for [^{3}H]quisqualic acid than previously reported for a similar construct produced from insect cells.

Materials and methods

Materials

Supersignal™ chemiluminescence substrate was purchased from Pierce & Warriner Ltd (Chester, UK). The Coomassie dye binding protein assay kit was obtained form Bio-Rad Laboratories Ltd (Hemel Hempstead, UK). Immunoblot detection was performed with horseradish peroxidase-coupled swine anti-rabbit serum from Dako Ltd (Ely, Cambridgeshire, UK). Immobilon-P polyvinylidene fluoride (PVDF) membranes were from Millipore Corporation (Bedford, MA, USA). Tissue culture materials and geneticin (G418 sulfate) were purchased from Invitrogen (UK). Talon™ metal affinity resin was obtained from BD Clontech (Basingstoke, UK). [^{3}H]quisqualic acid was purchased from Amersham (Buckinghamshire, UK), Ni-NTA superflow beads were from Qiagen Ltd. (UK). Quisqualate, *S*-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid (LY 367385), L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), and 2*S*,2′*R*,3′*R*-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) were from Tocris Cookson Ltd (Bristol, UK).

Methods

Construction of the truncated Human mGlu1 receptor N-terminal domain

The following primers 5′-CCGGAATTCGCCACCATGGTCGG-GCTCCTTTTGTTT-3′; 5′-CGCGGATCCTCAGTGGTGGTGGT-GGTGGTGGATGTTGCTCCACTCAAGATAGC-3′, were used to introduce a termination codon into the N-terminal domain of human mGlu1 receptor, just before the first putative transmembrane domain, at residue 592, by PCR. The latter of the primers included the codons for six histidines (underlined) to facilitate protein purification by metal-chelate chromatography. The resulting PCR product was verified by DNA sequencing and cloned into the *Eco*R1 and *Bam*H1 sites in the polylinker region of the mammalian expression vector pcDNA3 (Invitrogen, Paisley, UK). The protein product from expression of the construct corresponds to residues 1–592, including the putative signal peptide, of the N-terminal domain of mGlu1 receptor with a predicted molecular weight of 67 403 Da.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Discontinuous SDS–PAGE was performed using a 3% polyacrylamide stacking gel and 7.5 or 10% polyacrylamide running gels. Samples for electrophoresis were incubated with an equal volume of 2 × sample buffer 4% (w/v) SDS, 20% (v/v) glycerol, 100 mM Tris-HCl (pH 6.8), a trace of bromophenol blue and either with or without 20 mM dithiothreitol (DTT). For western blotting, proteins were transferred to PVDF membranes using a semi-dry transfer system. After blocking with 5% (w/v) dry non fat milk in phosphate-buffered saline containing 0.05% Tween-20, the membranes were incubated overnight at 4°C with affinity purified F1 antibody at dilutions of 2–6 μg/mL in blocking solution. This antibody, which is specific for the N-terminus of the mGlu1 receptor has been described previously (Ciruela and McIlhinney 1997). Immunoreactive bands were detected with swine anti-rabbit antibody conjugated to horseradish peroxidase followed by chemiluminescence detection using Supersignal™ substrate (Pierce & Warriner).

Production of the transfected cell line

Chinese hamster ovary (CHO) cells, maintained in RPMI medium containing 10% fetal calf serum were plated at 1.6×10^6 cells per 10 cm Petri dish 24 h prior to transfection. They were transfected using calcium phosphate precipitation of the 10 μg of DNA per dish (Jordan *et al*. 1996). After 48 h geneticin (1 mg/mL) was added to the medium and maintained in all subsequent cultures. After about 3 weeks isolated colonies were seen and picked for subculture.

These were screened for production of the protein by immunoblotting of the culture supernatant using the F1 antibody directed to the N-terminal region of mGlu1. Positive cultures were expanded, re-cloned twice by limiting dilution cloning and screened again for protein production. The strongest secreting clone, CHO-mGlu1.2, was expanded and used for this study.

Purification of the secreted protein and peptide sequencing
In order to produce medium for protein purification CHO-mGluR1.2 was subcultured into 18 × 10 cm Petri dishes at 1×10^6 cells per dish in RPMI 1640 medium containing L-glutamine 10% fetal bovine serum and penicillin/streptomycin. The cells were allowed to become confluent before the RPMI was replaced with AIMV medium containing 2 mM sodium butyrate, penicillin, streptomycin and L-glutamine (2 mM).

The cultures were left for 7 days before harvesting. The medium was pooled (150 mL) and dialysed against 50 mM Tris-HCl pH 8.0 (4 × 5 L). The dialysate was then rotated overnight at 4°C with 1.5 mL Talon^{TM} metal affinity resin. The resin was washed with 50 mM Tris-HCl pH 8.0 until the absorbance at 280 nm of the eluate was zero and then batch eluted with 1 mL aliquots of Tris buffer containing 100 mM imidazole. All fractions were analysed for the presence of the secreted N-terminus by SDS–PAGE followed by immunoblotting as described above.

Samples of fraction 1 (see Fig. 2) of the 100 mM eluate were then run and transferred to Immobilon-P PVDF membrane in the presence and absence of DTT. The protein was detected using Coomassie brilliant blue. Bands of interest were excised from the Coomassie blue-stained PVDF membrane and N-terminally sequenced using a PE-Applied Biosystems 494 A 'Procise' protein sequencer (Perkin Elmer, Applied Biosystems Division, UK).

Glycosidase treatment of cell lysates and supernatants
Cells (2×10^6) were pipetted from the flasks and washed three times in phosphate-buffered saline by centrifugation and resuspension. The cells were lysed in 2% aqueous SDS by heating to 100°C for 5 min. A protease inhibitor cocktail (Roche Diagnostics Ltd, Lewes, UK) was added and the lysate adjusted to contain 20 mM iodoacetamide. The culture supernatants were adjusted to 2% SDS, heated, protease inhibitors added, and adjusted to the same final iodoacetamide concentration. Equal aliquots of each (30 µL) were diluted to 150 µL final volume with either 50 mM sodium acetate buffer, pH 5 (EndoH) or 50 mM sodium phosphate, pH 7.2 (pNGaseF) containing 1% octylglucoside, protease inhibitors, and 1 U of either EndoH or pNGaseF. The reactions were incubated at 37°C for 16 h and samples analysed on 5% SDS–polyacrylamide gels under nonreducing conditions.

[^{3}H]Quisqualic acid binding
Secreted mGlu1 N-terminal protein was precipitated from harvested crude cell culture medium using an approximately 15 : 1 (v/v) ratio with Ni-NTA superflow beads by rolling for 2 h at 4°C. The mGlu1 N-terminal protein-bound beads were then precipitated by centrifugation at 10 000 *g* for 1 min and washed three times with 30 mL ice-cold assay buffer (20 mM HEPES, 2 mM $MgCl_2$, pH 7.4), re-centrifuging between each wash. After the final wash and spin the beads were re-suspended in assay buffer and incubated with approximately 1–50 nM [^{3}H]quisqualic acid for saturation binding or approximately 5 nM [^{3}H]quisqualic acid for competition binding, plus/minus competing drug, in a final volume of 0.5 mL on a shaker for 45 min at room temperature. Secreted protein from approximately 350 µL crude medium was used per tube and nonspecific binding was determined in the presence of 10 µM quisqualate. Bound and free ligand was separated by rapid filtration through Whatman GF/B filters on a 24 or 48 well Brandel cell harvester followed by 5 × 1 mL pulse-washes with ice-cold assay buffer. In experiments to determine the effect of Ca^{2+} on the affinity of agonist binding 1.3 mM $CaCl_2$ was included in the assay buffer.

For comparative purposes [^{3}H]quisqualic acid competition binding was also performed on membranes prepared [as previously described by Selkirk *et al*. (2001)] from baby hamster kidney (BHK) cells stably expressing the full-length mGlu1a receptor. These experiments used 50 µg per protein tube and approximately 10 nM [^{3}H]quisqualic acid. All other conditions remained the same.

Results

Characterization of the secreted protein

Following transfection of the truncated mGlu1 protein (the product of which is illustrated in Fig. 1) into the CHO cell

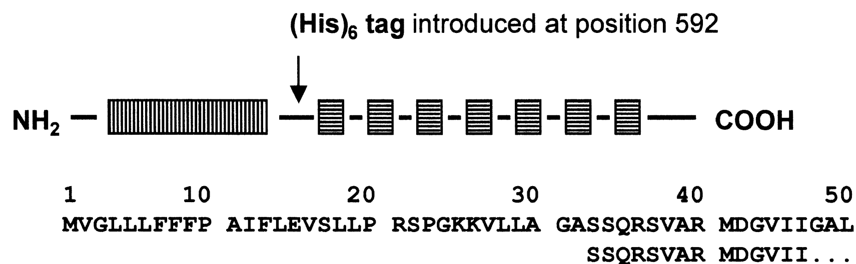

Fig. 1 Schematic representation of the N-terminal construct of human mGlu1 receptor. The vertically hatched box represents the N-terminus of the receptor and the horizontally hatched boxes indicate the transmembrane domains of the protein. A PCR mutagenesis reaction was used to introduce six histidine residues followed by a stop codon at a position corresponding to residue 592 in the human mGlu1 sequence, just before the first proposed transmembrane domain. The predicted protein sequence deduced from the first start codon in the human cDNA is shown up to 50 residues (upper text) and the sequence obtained from the purified secreted N-terminal domain is shown below (underlined). Clearly there has been a specific cleavage at residue 32 to yield the secreted product.

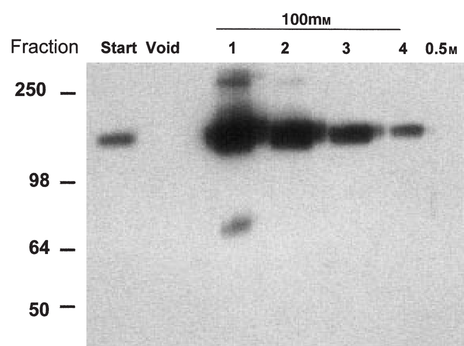

Fig. 2 Purification of the secreted mGlu1 receptor domain. Cell culture medium from CHO-mGlu1.2 was rotated with Talon™ resin as described in Materials and methods. Aliquots (25 μL) of the original culture medium (start) and the supernatant after Talon™ treatment (void) were analysed by immunoblotting on a 7.5% SDS–polyacrylamide gel. Samples (10 μL) of the different 100 mM imidazole eluates (1–4) and the final 500 mM imidazole (0.5 M) eluate were also analysed, and the immunoblot reacted with a rabbit N-terminal mGlu1 receptor-specific antibody. The bands were detected using ECL following reaction with a swine horseradish peroxidase-conjugated anti-rabbit antibody. The molecular weight markers are SeeBlue™ prestained standards and are; 250 kDa myosin; 98 kDa bovine serum albumin; 64 kDa glutamic dehydrogenase; 50 kDa, alcohol dehydrogenase.

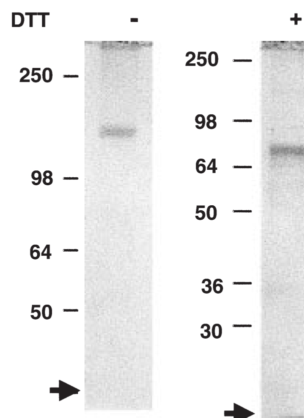

Fig. 3 Analysis of the purified, secreted protein by SDS–PAGE. Samples (25 μL; 1.25 μg) of the fraction 1 (Fig. 1) were analysed either with (+) or without (–) treatment with dithiothreitol (DTT, 20 mM) and the gels stained with Coomassie blue. Following DTT treatment the apparent molecular weight of the purified protein was reduced from 130 kDa to 75 kDa, suggesting that it was secreted as a disulphide-bonded dimer. The molecular weight markers are as in Fig. 2 with carbonic anhydrase (36 kDa) and myoglobin (30 kDa) resolved on the higher strength gel. The arrow indicates the position of the dye front on both gels.

line, and selection with geneticin, 20 resistant colonies were isolated of which only three secreted detectable quantities of the N-terminal domain protein as determined by immunoblotting with an N-terminal-specific anti-mGlu1 receptor antibody. The colony giving the highest level of secreted N-terminal protein was re-cloned twice and one isolate termed CHO-mGluR1.2 expanded and used in this study. Cells were grown to high density and the medium (150 mL) pooled for purification. Following rotation with Talon™ metal-chelate resin the immunoreactive protein, which migrated with an apparent molecular weight of 130 kDa under nonreducing conditions on SDS–polyacrylamide gels, was completely removed from the culture medium (see Fig. 2; compare 'start' and 'void'). Elution of the protein from the washed resin with 100 mM imidazole resulted in the recovery of immunoreactive protein in four fractions. These were all enriched for the protein with the first containing the greatest amount of the N-terminal domain. Elution of the resin with high concentrations of imidazole (500 mM) after the fourth fraction did not result in the release of further protein (Fig. 2; 0.5 M).

Analysis of the purified protein under nonreducing and reducing conditions revealed that it was secreted as a disulphide-bonded dimer since treatment with 20 mM DTT resulted in a change in migration from an apparent molecular weight from 130 kDa to 75 kDa (Fig. 3). Therefore, the smaller molecular weight bands seen in fraction 1 may represent a small amount of nondimerized N-terminal domain, released from the cells during culture, or degraded material. These gels also showed that the protein was highly purified following the Talon™ metal-chelate chromatography and the yield of purified protein has varied from 0.2 to 0.4 mg/L in the culture supernatant from different cultures. Following transfer of the purified product to PVDF membranes the bands from both reduced and unreduced samples were subjected to N-terminal peptide sequencing. The results from both bands were identical and yielded the sequence underlined in Fig. 1. This is compared with the predicted open reading frame deduced from the cDNA sequence of the human mGlu1 receptor (Fig. 1; upper text). As can be seen, the secreted protein has a sequence identical to the predicted cloned sequence starting at residue 33. The sequence obtained gave no evidence of 'ragged' ends, suggesting that the preceding residues are cleaved from the newly synthesized protein as a signal peptide, rather than by nonspecific degradation of the product during either secretion or purification.

Endoglycosidase analysis of the secreted protein

In order to confirm that the secreted material was glycosylated, samples of the supernatant and cell lysate from the CHO cells were subjected to deglycosylation with pNGaseF or EndoH, and the digests analysed on SDS-polyacrylamide gels. The results show that the secreted dimeric form of the mGlu1 N-terminal domain is fully EndoH-resistant as is the native receptor (Chan *et al.* 2001) and has an apparent molecular weight of 130 kDa suggesting that it is fully

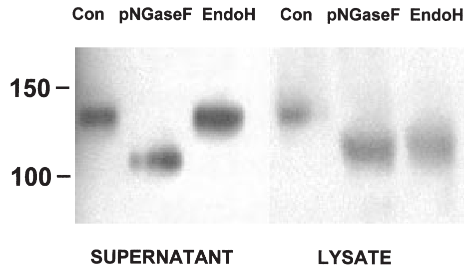

Fig. 4 Endoglycosidase treatment of mGlu1 N-terminal protein secreted into the culture medium, or in cell lysates. Cell lysates, or the culture medium, were treated with the either pNGaseF or EndoH as described in the Materials and methods. Samples of the enzyme digests were analysed unreduced on 5% SDS–polyacrylamide gels and immunoblotted using a rabbit N-terminal mGlu1 receptor-specific antibody. Only the cell lysate-derived material shows EndoH sensitivity, as revealed by the increase in gel mobility, indicating that it does not contain mature polysaccharide groups.

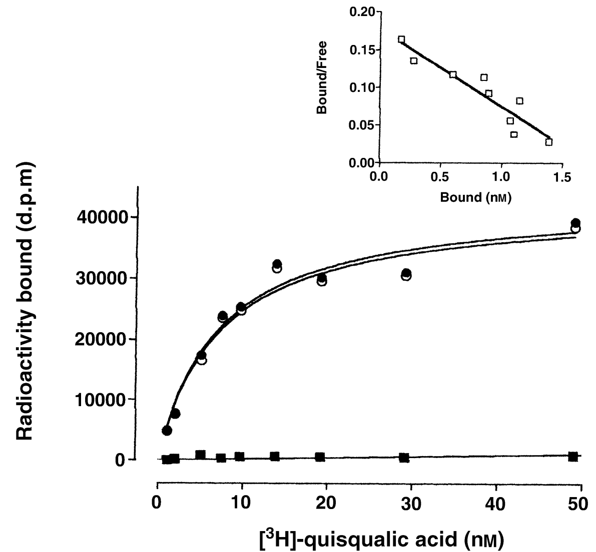

Fig. 5 [^{3}H]Quisqualic acid saturation-binding to the secreted mGlu1 N-terminal domain. Secreted protein from approximately 350 μL crude medium was precipitated (2 h, 4°C) with approximately 25 μL Ni-NTA superflow beads per tube and incubated for 45 min at room temp with approximately 1–50 nM [^{3}H]quisqualic acid. Incubations were terminated as described in the Materials and methods section and total binding (●), nonspecific binding (■) and specific binding (○) determined. A representative experiment is shown performed in duplicate that has been repeated with similar results on four separate occasions.

glycosylated and contains branched carbohydrate chains (Fig. 4). However, the majority of the intracellular material was EndoH-sensitive with an apparent molecular weight of 115 kDa, indicating that it was not fully glycosylated (Fig. 4). It should be noted that both the secreted and intracellular proteins formed disulphide-bonded dimers.

Quantitation and pharmacology of the secreted protein

The secreted mGlu1 receptor N-terminal domain was assessed by saturation binding analysis using [^{3}H]quisqualic acid (Fig. 5). B_{max} values varied between different batches of crude cell culture medium generating values in the range 0.22–2.4 pmol/mL original crude culture medium. The affinity of [^{3}H]quisqualic acid for the mGlu1 N-terminal domain however, remained consistent between batches generating a K_d of 5.6 ± 1.8 nM ($n = 4$), which is approximately two-fold lower than that observed in cell membranes prepared from BHK-mGlu1α cells [$K_d = 10 \pm 1$ nM (Selkirk *et al.* 2001)].

The pharmacology of the secreted protein, assessed by [^{3}H]quisqualic acid competition binding, closely resembles that of the mGlu1α receptor. L-Glutamate, the group I mGlu receptor-selective agonist quisqualate and the mGlu1 receptor-selective antagonist LY 367385 all completely displaced [^{3}H]quisqualic acid binding to generate K_i values of 280 ± 116, 2.1 ± 0.3, and 53.0 ± 7.2 nM, respectively (Fig. 6a). These K_i values were lower than those determined using the full-length receptor expressed in cell membranes prepared from BHK-mGlu1α cells [K_is (nM), quisqualate, 3.3 ± 0.2; LY 367385, 459 ± 49]. Furthermore, [^{3}H]quisqualic acid binding was displaced only at very high concentrations by the group-II and -III selective mGlu receptor agonists DCG-IV and L-AP4 [DCG-IV 10 μM, 19 ± 5%; L-AP4 (300 μM), 20 ± 5% inhibition of specific binding, respectively].

The influence of a physiological level of [Ca^{2+}] (1.3 mM) on agonist binding affinities was also assessed with respect to L-glutamate and quisqualate displacement of [^{3}H]quisqualic acid binding (Fig. 6b). The presence and absence of Ca^{2+} was found not to significantly influence the displacement isotherms generated for either agonist (K_i values (nM) – Ca^{2+}, 250 ± 28 and 1.9 ± 0.5; + Ca^{2+}, 180 ± 47 and 2.8 ± 0.7, for L-glutamate and quisqualate, respectively). Preliminary experiments on the full-length receptor expressed in cell membranes prepared from BHK-mGlu1α cells also indicate that the presence or absence of millimolar Ca^{2+} does not significantly affect the ability of either quisqualate or LY 367385 to displace [^{3}H]quisqualic acid binding (data not shown).

Discussion

The results presented here show that the N-terminal domain of human mGlu1 receptor is secreted as a disulphide-bonded dimer, as previously suggested (Romano *et al.* 1996), and as found for the same N-terminal domain and that of the mGlu4 receptor secreted from insect cells (Okamoto *et al.* 1998; Han and Hampson 1999; Tsuji *et al.* 2000). The fact that both the 75 kDa and 130 kDa proteins yield the same sequence is conclusive evidence that the former is a disulphide-bonded form of the latter

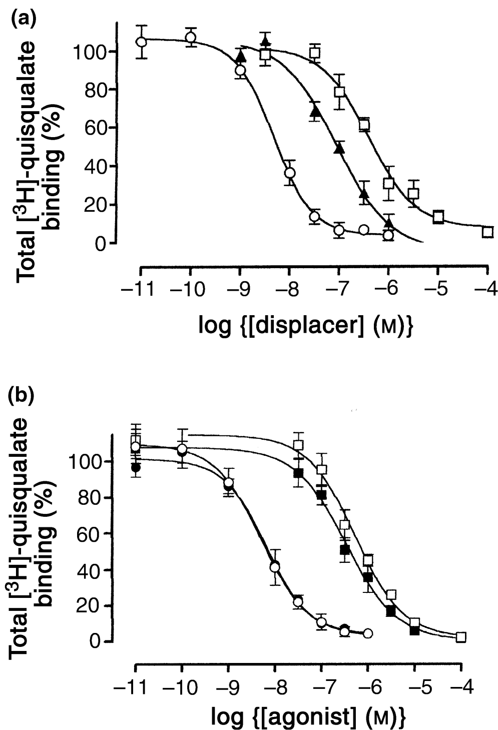

Fig. 6 [^{3}H]Quisqualic acid competition-binding analysis, and effect of Ca^{2+} on agonist binding to the secreted mGlu1 N-terminal domain. Secreted protein from approximately 350 μL crude medium was precipitated (2 h, 4°C) with approximately 25 μL Ni-NTA superflow beads per tube and incubated with approximately 5 nM [^{3}H]quisqualic acid. In (a) incubations were conducted for 45 min at room temperature ± each competing compound. Displacement isotherms are shown for quisqualate (○), L-glutamate (□) and LY 367385 (▲). In panel (b) competition binding was performed in either normal assay medium (open symbols) or medium supplemented with 1.3 mM Ca^{2+} (closed symbols) for quisqualate (circles) or L-glutamate (squares). Incubations were terminated as described in the Materials and methods section. Non-specific binding was determined in the presence of 10 μM quisqualate. Data are expressed as percentage total [^{3}H]quisqualic acid bound and are presented as means ± SEM for between four and six (a) or three (b) individual experiments.

and the sequence data also confirm that the secreted protein is indeed the N-terminal domain of the mGlu1 receptor. In addition, the site of signal peptide cleavage reported here, between residues 32 and 33, is identical to that found in the protein secreted from the insect cells (Tsuji *et al.* 2000). Interestingly, endoglycosidase treatment of the secreted N-terminal dimer showed that it was predominantly EndoH-resistant, whereas the intracellular dimeric N-terminal domain was EndoH-sensitive. This provides strong evidence for dimerization of the receptor taking place in the endoplasmic reticulum, before the maturation of the carbohydrate side chains, and is consistent with earlier evidence using brefeldin-A to show that dimerization was initiated in the endoplasmic reticulum (Robbins *et al.* 1999).

From the B_{max} values obtained from the binding studies on the culture supernatants, where the average binding was 1.15 pmol/mL, one would expect 172 μg/L of protein, if both binding sites in the dimer were occupied. This is somewhat lower than the average yield of purified protein of 300 μg/L. However, we note that in other studies on the binding properties of soluble glutamate receptor binding domains only 40–50% of the maximal binding was observed (Kuusinen *et al.* 1995; Tsuji *et al.* 2000). This would be consistent with the data found here and has been interpreted as either due to the presence of a proportion of unfolded and hence unbound protein, or an inability of the ligand to saturate fully the soluble, dimeric protein. Consequently, the binding studies may have underestimated the total yield of protein. It is difficult to compare the yields for the soluble domain obtained from the mammalian cells with those obtained from the insect cells, since these were not given. However, estimates based on the reported binding of agonist in the culture supernatants of the latter, suggest that the mammalian cells are at least as efficient as the insect cells in producing properly folded secreted protein.

The pharmacology of the soluble protein was similar to that of the full receptor expressed in BHK cell membranes, with the soluble protein generally displaying a higher affinity for the agonists than the membrane-bound, full-length receptor. This is a pattern also noted for the mGlu1 receptor soluble domains and for the mGlu4 receptor (Okamoto *et al.* 1998; Han and Hampson 1999), and may reflect the absence of the transmembrane domains and/or other interactions in the full receptor with heterotrimeric G proteins that might modulate agonist binding. In contrast to the apparently lower affinity of the soluble domain of mGlu4 receptor for antagonists when compared to the native mGlu4 receptor, the soluble domain of mGlu1 described here demonstrated an eight-fold greater affinity for the antagonist LY 367385 than the full receptor. One other striking finding is that the affinity of the soluble mGlu1 protein for [^{3}H]quisqualic acid reported here is significantly higher than that produced from the insect cell-derived material – 5.6 nM as against 38 nM (Okamoto *et al.* 1998). In addition, the full-length receptor produced in insect cells also had a lower K_i for quisqualate than that expressed in BHK cells reported here, 30 nM as compared with 10 nM. Thus, this may reflect the more optimal processing of the proteins by the mammalian cells, for example in terms of receptor glycosylation. With respect to the latter cotranslational modification it is interesting to note that Kunishima *et al.* (2000) have reported that the insect cell-derived soluble N-terminal domain is N-glycosylated at two sites (N^{98} and N^{223}). It is possible that the carbohydrate moieties at these positions may be different when generated by an insect expression system compared to the (EndoH resistant) modification demonstrated in the present study. In

addition, the truncated mGlu1 protein possesses two further potential N-glycosylation sites (N^{397}, N^{515}), and it will be important to establish whether glycosylation at these sites occurs in mammalian expression systems and whether such modification(s) influences interactions of agonists and competitive antagonists with the receptor.

Very recently, we have generated single and combined point mutations of each of the four N-terminal domain N-glycosylation consensus sites in the full-length human mGlu1α receptor. Preliminary analysis of these mutants transiently expressed in either CHO-K1 or HEK293 cells suggests that introduction of N to Q mutations at ^{397}N and ^{515}N, alone or additional to mutation of ^{98}N and ^{223}N, affects receptor electrophoretic mobility, cell-surface expression, and the ability of the receptor to couple to G protein/effector systems (J.V. Selkirk, G.W. Price, S.R Nahorski and R.A.J. Challiss, unpublished results). Such data clearly point to the possibility that the N-terminal domain of human mGlu1 receptor may be cotranslationally processed differently by insect and mammalian cell systems.

A number of studies have demonstrated an apparent sensitivity of the mGlu1 receptor to changes in Ca^{2+} concentration in the millimolar range (Kubo *et al*. 1998; Saunders *et al*. 1998), suggesting that Ca^{2+} *per se* (Kubo *et al*. 1998) may activate the mGlu1 receptor, or that Ca^{2+} can positively modulate agonist-mediated mGlu1 receptor activation (Saunders *et al*. 1998). Enhanced receptor function in the presence of Ca^{2+}_e has also been shown for the $GABA_B$ receptor, both in recombinant cell systems and, to a greater extent, in rat cortical cell membranes, and this has been shown to be agonist-dependent, with the natural ligand GABA demonstrating a greater potentiation than the synthetic agonist baclofen (Wise *et al*. 1999). The demonstration that the Ca^{2+}-sensing receptor responds not only to changes in Ca^{2+}_e, but also to aromatic L-amino acids (Conigrave *et al*. 2000) provides further evidence for the proposal that coagonism might be a common motif of the family C G protein-coupled receptors. The generation of the N-terminal mGlu1 receptor domain provides an ideal opportunity to assess the Ca^{2+} requirement for optimal agonist binding. Here we report that the interaction of both quisqualate and L-glutamate with the N-terminal protein is unaffected by the presence of a physiological Ca^{2+} concentration. Thus, at the level of agonist binding the mGlu1 receptor does not appear to act as a Ca^{2+} sensor. This finding is in agreement with a recent study, which also suggests that any effect of Ca^{2+}_e on mGlu1 receptor signalling is most likely to be indirect (Nash *et al*. 2001).

In conclusion, this study has shown that mammalian expression systems can produce significant quantities of soluble domains of mGlu receptors and that these provide useful tools for the characterization of the ligand binding sites. In addition the ligand binding characteristics of the mammalian derived mGlu1 N-terminus differs significantly from that produced in insect cells, and it will therefore, be interesting to compare their structures.

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References

Chan W. Y., Soloviev M. M., Ciruela F. and McIlhinney R. A. J. (2001) Molecular determinants of metabotropic glutamate receptor 1B trafficking. *Mol. Cell. Neurosci.* **17**, 577–588.

Ciruela F. and McIlhinney R. A. J. (1997) Differential internalisation of mGluR1 splice variants in response to agonist and phorbol esters in permanently transfected BHK cells. *FEBS Lett.* **418**, 83–86.

Conigrave A. D., Quinn S. J. and Brown E. M. (2000) L-Amino acid sensing by the extracellular Ca^{2+}-sensing receptor. *Proc. Natl Acad. Sci. USA* **97**, 4814–4819.

Conn P. J. and Pin J. P. (1997) Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol.* **37**, 205–237.

Han G. M. and Hampson D. R. (1999) Ligand binding to the amino-terminal domain of the mGluR4 subtype of metabotropic glutamate receptor. *J. Biol. Chem.* **274**, 10008–10013.

Jordan M., Schallhorn A. and Wurm F. M. (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium phosphate precipitate formation. *Nucleic Acid Res.* **24**, 596–601.

Knöpfel T. and Gasparini F. (1996) Metabotropic glutamate receptors: potential drug targets. *Drug Discov. Today* **1**, 103–108.

Kubo Y., Miyashita T. and Murata Y. (1998) Structural basis for a Ca^{2+}-sensing function of the metabotropic glutamate receptors. *Science* **279**, 1722–1725.

Kunishima N., Shimada Y., Tsuji Y., Sato T., Yamamoto M., Kumasaka T., Nakanishi S., Jingami H. and Morikawa K. (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **407**, 971–977.

Kuusinen A., Arvola M. and Keinanen K. (1995) Molecular dissection of the agonist binding site of an AMPA receptor. *EMBO J.* **14**, 6327–6332.

Nash M. S., Saunders R., Young K. W., Challiss R. A. J. and Nahorski S. R. (2001) Reassessment of the Ca^{2+}-sensing property of a type I metabotropic glutamate receptor by simultaneous measurement of inositol 1,4,5-trisphosphate and Ca^{2+} in single cells. *J. Biol. Chem.* **276**, 19286–19293.

O'Hara P. J., Sheppard P. O., Thogersen H., Venezia D., Haldeman B. A., McGrane V., Houamed K. M., Thomsen C., Gilbert T. L. and Mulvihill E. R. (1993) The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron* **11**, 41–52.

Okamoto T., Sekiyama N., Otsu M., Shimada Y., Sato A., Nakanishi S. and Jingami H. (1998) Expression and purification of the extra-

cellular ligand binding region of metabotropic glutamate receptor subtype 1. *J. Biol. Chem.* **273**, 13089–13096.
Pin J. P. and Duvoisin R. (1995) The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* **34**, 1–26.
Robbins M. J., Ciruela F., Rhodes A. and McIlhinney R. A. J. (1999) Characterization of the dimerization of metabotropic glutamate receptors using an *N*-terminal truncation of mGluR1α. *J. Neurochem.* **72**, 2539–2547.
Romano C., Yang W. L. and O'Malley K. L. (1996) Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J. Biol. Chem.* **271**, 28612–28616.
Saunders R., Nahorski S. R. and Challiss R. A. J. (1998) A modulatory effect of extracellular Ca^{2+} on type 1α metabotropic glutamate receptor-mediated signalling. *Neuropharmacology* **37**, 273–276.
Selkirk J. V., Price G. W., Nahorski S. R. and Challiss R. A. J. (2001) Cell type-specific differences in the coupling of recombinant mGlu1α receptors to endogenous G protein sub-populations. *Neuropharmacology* **40**, 645–656.
Takahashi K., Tsuchida K., Tanabe Y., Masu M. and Nakanishi S. (1993) Role of the large extracellular domain of the metabtropic glutamate receptors in agonist selectivity. *J. Biol. Chem.* **268**, 19341–19345.
Tanabe Y., Masu M., Ishii T., Shigemoto S. and Nakanishi S. (1992) A family of metabotropic glutamate receptors. *Neuron* **8**, 169–179.
Tones M. A., Bendali H., Flor P. J., Knöpfel T. and Kuhn R. (1995) The agonist selectivity of a class III metabotropic glutamate receptor, human mGluR4a, is determined by the *N*-terminal extracellular domain. *Neuroreport* **7**, 117–120.
Tsuji Y., Shimada Y., Takeshita T., Kajimura N., Nomura S., Sekiyama N., Otomo J., Usukura J., Nakanishi S. and Jingami H. (2000) Cryptic dimer interface and domain organization of the extracellular region of metabotropic glutamate receptor subtype 1. *J. Biol. Chem.* **275**, 28144–28151.
Wise A., Green A., Main M. J., Wilson R., Fraser N. and Marshall F. H. (1999) Calcium-sensing properties of the $GABA_B$ receptor. *Neuropharmacology* **38**, 1647–1656.