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Interactions of the C Terminus of Metabotropic Glutamate Receptor Type 1α with Rat Brain Proteins: Evidence for a Direct Interaction with Tubulin

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Abstract: Metabotropic glutamate receptors (mGluRs) are coupled to G protein second messenger pathways and modulate glutamate neurotransmission in the brain, where they are targeted to specific synaptic locations. As part of a strategy for defining the mechanisms for the specific targeting of mGluR1α, rat brain proteins which interact with the intracellular carboxy terminus of mGluR1α have been characterized, using affinity chromatography on a glutathione *S*-transferase fusion protein that contains the last 86 amino acids of mGluR1α. Three of the proteins specifically eluted from the affinity column yielded protein sequences, two of which were identified as glyceraldehyde-3-phosphate dehydrogenase and β-tubulin; the other was an unknown protein. The identity of tubulin was confirmed by western immunoblotting. Using a solid-phase binding assay, the mGluR1α–tubulin interaction was shown to be direct, specific, and saturable with a K_D of 2.3 $\pm$ 0.4 μM. In addition, mGluR1α, but not mGluR2/3 or mGluR4, could be coimmunoprecipitated from solubilized brain extracts with tubulin using anti-β-tubulin antibodies. However, mGluR1α could not be coimmunoprecipitated with the tubulin binding protein gephyrin, nor could it be coimmunoprecipitated with PSD95. Collectively these data demonstrate that the last 86 amino acids of the carboxyl-terminal tail of mGluR1α are sufficient to determine its interaction with tubulin and that there is an association of this receptor with tubulin in rat brain. **Key Words:** Metabotropic—Glutamate receptor—Tubulin—Targeting.
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Metabotropic glutamate receptors (mGluRs) constitute a family of large G protein-coupled receptors that show little sequence homology with the superfamily of smaller G protein-linked receptors (Tanabe et al., 1992). Eight members of the mGluR family have been identified, several of which generate different subtypes by alternative mRNA splicing (Knopfel et al., 1995; Pin and Duvoisin, 1995). These eight mGluR receptors and their subtypes may be categorized into three subgroups on the basis of their sequence homology, agonist selectivity, and signal transduction pathway. The first group contains mGluR1 and mGluR5, which share the strongest sequence homology (62% identical at the amino acid level), are coupled to phospholipase C in transfected cells, and have quisqualic acid as their most potent agonist. The second group consists of mGluR2 and mGluR3, which couple negatively to adenyl cyclase in transfected cells and for which L-2-(carboxycyclopropyl) glycine is the most potent agonist. The third group contains mGluR4, mGluR6, mGluR7, and mGluR8, which also couple negatively to adenyl cyclase but have L-2-amino-4-phosphonobutyric acid as their most potent agonist.

Splice variants for mGluR1, mGluR4, mGluR5, and mGluR7 have been described, which involve alterations in their C-terminal sequences (Pin et al., 1992; Simoncini et al., 1993; Minakami et al., 1994; Flor et al., 1997). Four C-terminal splice variants of mGluR1 have been described, namely, mGluR1α, mGluR1β, mGluR1c, and mGluR1d (Pin et al., 1992; Knopfel et al., 1995). In mGluR1β, the insertion of an 85-base sequence in the carboxy-terminal tail introduces a stop codon that results in a 318-amino acid deletion and also changes the reading frame, resulting in a different and much shortened C-terminal amino acid sequence from that of mGluR1α. Similarly, in mGluR1c and mGluR1d a different insertion at the same position results in further changes in the sequence of the C-terminal tails, which are also much smaller than that of mGluR1α. An additional splice variant of mGluR1, mGluR1e, is known that appears to result in the production of the N terminus of the protein only (Pin and Duvoisin, 1995).

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Abbreviations used: BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; mGluR, metabotropic glutamate receptor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-Ca/Mg, phosphate-buffered saline containing 0.8 m*M* $CaCl_2$ and 0.4 m*M* $MgCl_2$; PBS-N, phosphate-buffered saline containing 0.05% Nonidet P-40; PSD, postsynaptic density; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate.

Immunocytochemical studies have shown that the different groups of the mGluRs exhibit differential intraneuronal targeting, with the group 1 mGluRs being predominantly postsynaptic, whereas the group 2 and group 3 receptors are generally presynaptic (Baude et al., 1993; Ohishi et al., 1995; Shigemoto et al., 1996, 1997). The group 2 and group 3 mGluRs can be differentially targeted to different regions of axons, because in the rat hippocampus the former are concentrated in the preterminal regions of axons, whereas the latter were found predominantly in the presynaptic active zones (Shigemoto et al., 1997). Furthermore, the precise synaptic location of mGluR1α and mGluR5 is highly ordered, with the protein present in an annulus that surrounds the postsynaptic density (PSD) (Baude et al., 1993; Lujan et al., 1996, 1997). One possible explanation for the differential neuronal and synaptic targeting of the different mGluRs is that this is directed by interactions of their cytoplasmic C termini with specific targeting proteins. Precisely, this type of targeting mechanism appears to operate for the synaptic localization of the ionotropic glutamate receptors, and several different proteins, containing PDZ domains, have been described that interact with specific C-terminal sequences of these receptors (Hunt et al., 1996; Kim et al., 1996; Muller et al., 1996; Niethammer et al., 1996; Dong et al., 1997). A similar PDZ domain-containing protein, which also binds to the C-terminal residues of mGluR1α and mGluR5, called HOMER, has been described, although its precise function is currently unclear (Brakeman et al., 1997).

To identify other proteins that interact with the C terminus of mGluR1α and could be candidates for its targeting or localization, we have used affinity chromatography of proteins from rat brain on a column containing the last 86 residues of the receptor. In this report we identify one such protein as tubulin and show that it interacts directly with mGluR1α.

MATERIALS AND METHODS

Generation of fusion proteins and antibodies

Two antisera against mGluR1 were used in this study, designated as F1 and F2. They were raised against a histidine-tagged fusion protein containing an amino-terminal sequence of mGluR1, residues 121–341 (F1 protein) (Ciruela and McIlhinney, 1997), or a histidine-tagged fusion protein containing the last 86 amino acids of mGluR1α carboxyl-terminal tail, residues 1,114–1,199 (F2 protein). The latter was produced by cloning a *Pst*I–*Pst*I fragment of pmGR1 (Masu et al., 1991) into the bacterial expression vector pET-28c(+) (Novagen). The same fragment was also subcloned into the pGEX-5X-2 bacterial expression vector (Pharmacia Biotech) to produce a fusion protein termed F2-GST. The last 66 amino acids of mGluR4 were also cloned into the vector pGEX4T-1 (Pharmacia Biotech) to produce the fusion protein M4-GST. The production and purification of the fusion proteins, the immunization of rabbits, and affinity purification of the antisera were performed as described previously (Ciruela and McIlhinney, 1997; Clark et al., 1998). The sequence of the cDNAs and their orientation in the vector were confirmed by DNA sequencing. The anti-mGluR4 has been described previously (Alaluf et al., 1995), and the mGluR2/3 (AB1553) antibody was purchased from Chemicon International.

Preparation of synaptosomes and tissue samples

Synaptosomes were prepared as described earlier (Hajos and Csillag, 1976). In brief, five adult rats were decapitated, and the whole brain was dissected, cleaned of white matter, and homogenised in 0.32 *M* sucrose (10 volumes). The homogenate was centrifuged twice at 1,500 *g* for 10 min each. The combined supernatants (S_1) were then centrifuged at 9,500 *g* for 20 min. The resulting supernatant (S_2) and pellet (P_2 membranes) were carefully separated. S_2 was then centrifuged at 80,000 *g* for 90 min to pellet insoluble materials. The resulting supernatant was filtered (pore size, 0.4 μm; Millipore) and diluted 1:2 in 2× phosphate-buffered saline (PBS) containing 1.6 m*M* $CaCl_2$ and 0.8 m*M* $MgCl_2$ to give the soluble fraction S_3. P_2 membranes were resuspended in 10 ml of 0.32 *M* sucrose and layered over 20 ml of 0.8 *M* sucrose. Following centrifugation for 25 min at 9,500 *g* the synaptosomes were collected at the upper interface of the two sucrose solutions.

Protein content was determined using the assay based on bicinchoninic acid obtained from Pierce (Sorensen and Brodbeck, 1986).

Chromatography of S_3 fraction using a glutathione *S*-transferase (GST) or F2-GST affinity column and microsequencing

Affinity columns were prepared by direct coupling of 2 mg of fusion proteins to 1 *g* of cyanogen bromide-activated Sepharose as described by Trotta et al. (1979). The soluble extract S_3 was sequentially passed over a GST and then an F2-GST affinity column (2 ml) at 4°C in PBS containing 0.8 m*M* $CaCl_2$ and 0.4 m*M* $MgCl_2$ (PBS-Ca/Mg). The columns were washed with 15 volumes of PBS-Ca/Mg until no protein was detected in the eluates. Elution of bound proteins was performed with 0.1 *M* triethylamine (pH 11.5) and monitored by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and silver staining. To allow microsequencing of proteins, the samples containing specifically eluted proteins were pooled together and freeze-dried. Samples were analysed on 10% SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) essentially as described by Matsudaira (1987). Bands of interest were excised from the Coomassie Blue-stained PVDF membrane and N-terminally sequenced using a PE-Applied Biosystems model 494A Procise protein sequencer (Perkin Elmer, Applied Biosystems Division, Warrington, U.K.).

SDS-PAGE immunoblotting and overlay experiments

SDS-PAGE was performed on 7–10% gels using a discontinuous Tris-glycine buffer system (Laemmli, 1970). Immunoblotting was performed as described previously (Ciruela and McIlhinney, 1997). Incubations with the primary antibodies were performed overnight at 4°C using the following antibody concentrations: affinity-purified F2 antibody (F2-Ab; 2–4 μg/ml), monoclonal anti-β-tubulin antibody (1:200; Sigma), monoclonal anti-gephyrin antibody (1:500; Alexis Corp.), monoclonal anti-PSD95 antibody (1:500; Transduction Laboratories), anti-mGluR4 (1:2,000), or anti-mGluR2/3 (1:2,000). Immunoreactive bands were visualized using a 2-h incubation with either swine anti-rabbit or sheep anti-mouse antibody conjugated to horseradish peroxidase at room temperature, followed by chemiluminescence detection (Pierce Supersignal substrate).

For overlay experiments with the fusion proteins the SDS-PAGE-separated proteins were transferred to PVDF membranes as for immunoblotting. After blocking with 5% (wt/vol) dry milk in PBS containing 0.05% Nonidet P-40 (PBS-N), PVDF membranes were washed in cold PBS-N and incubated overnight at 4°C with the fusion proteins, either GST alone or F2-GST. These were used at a final concentration of 3 μM in PBS-Ca/Mg containing 0.05% Nonidet P-40 and 5% (wt/vol) dried milk. The membranes were washed (3 × 10 min) with PBS-N-Ca/Mg and incubated with goat anti-GST antibody (1:2,000; Pharmacia Biotech) for 4 h at 4°C. Immunoreactive bands were detected using rabbit anti-goat antibody conjugated to horseradish peroxidase followed by chemiluminescence detection.

Immunoprecipitation of proteins

Supernatant S_1 from whole rat brain was solubilised in ice-cold lysis buffer (PBS-Ca/Mg) containing 1% (vol/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholic acid, and 0.1% (wt/vol) SDS for 1 h on ice. The solubilized preparation was then centrifuged at 80,000 *g* for 90 min. The supernatant (1 mg of protein/ml) was processed for immunoprecipitation, each step of which was conducted with constant rotation at 0–4°C. All samples were precleared by incubation overnight with preimmune rabbit IgG (40 μg/ml). Then 80 μl of a 50% (vol/vol) suspension of protein A-Sepharose beads was added, and the mixture was incubated for a further 6 h. After the beads were pelleted at 10,000 *g* for 15 s, the supernatant was transferred to a clean tube containing one of the following antibodies: affinity-purified anti-mGluR1 (F1-Ab; 10 μg/ml), monoclonal anti-β-tubulin (5 μg/ml), monoclonal anti-gephyrin (5 μg/ml), monoclonal anti-PSD95 (5 μg/ml), anti-mGluR2/3 (15 μg/ml), or anti-mGluR4 (10 μg/ml). After overnight incubation 80 μl of protein A-Sepharose beads was added and then rotated for 6 h as above. The beads were washed twice with ice-cold lysis buffer, twice with ice-cold lysis buffer containing 0.1% (vol/vol) Nonidet P-40, 0.05% (wt/vol) sodium deoxycholic acid, and 0.01% (wt/vol) SDS, and once with ice-cold PBS-Ca/Mg and aspirated to dryness with a 28-gauge needle. Subsequently, 60 μl of SDS-PAGE sample buffer was added to each sample. Immune complexes were dissociated by heating to 100°C for 5 min and resolved by SDS-PAGE in 7% gels. The gels were electrophoresed and immunoblotted as described above.

Tubulin preparations

Microtubule proteins were prepared by the method of Vallee (1986). In brief, soluble extracts (S_3) obtained from rat brain were dialysed in PEM buffer [0.1 *M* PIPES-NaOH (pH 6.6) containing 1 m*M* EGTA and 1 m*M* $MgSO_4$]. Microtubules were polymerised by addition of 1 m*M* GTP and warmed to 37°C for 30 min. After polymerization, microtubules were pelleted by centrifugation at 15,000 *g* for 30 min at 37°C through a sucrose gradient (PEM buffer containing 10% sucrose and 1 m*M* GTP). The microtubules were resuspended by addition of 20 ml of ice-cold PEM buffer containing 1 m*M* GTP. The mixture was homogenized in a Teflon/glass homogenizer, incubated on ice for 30 min to depolymerize the microtubules, and then centrifuged at 15,000 *g* for 30 min at 2°C. A second microtubule assembly–disassembly cycle was performed. Separation of tubulin from microtubule-associated proteins was performed using diethylaminoethyl-Sephadex (Pharmacia) chromatography (Vallee, 1986). Tubulin was eluted with PEM buffer containing 0.5 *M* NaCl and 0.1 m*M* GTP, dialysed in sodium carbonate buffer (pH 9.0) overnight, and used for solid-phase binding assay. The resulting preparation was analysed by SDS-PAGE and Coomassie Blue staining. The tubulin concentration was determined from densitometric scans of gels stained with Coomassie Blue. Bovine serum albumin (BSA) was used for calibration.

Solid-phase binding assay and analysis of binding data

The affinity-purified F2-GST fusion protein was radiolabelled with ^{125}I by the chloramine-T method described by Daddona and Kelley (1978) to a specific activity of 1 μCi/μg and then used in microtitre plate assay to determine its binding to immobilized tubulin. In brief, microtitre wells were coated with 8 μg per well of tubulin or control protein (BSA) in sodium carbonate buffer (pH 9.0) overnight at 4°C. Before protein binding, the wells were blocked with 1% BSA in PBS-Ca/Mg for 1 h and then incubated with ^{125}I-labelled F2-GST fusion protein at various concentrations in PBS-Ca/Mg containing 1% BSA overnight at 4°C. At the end of the incubation period free ^{125}I-labelled F2-GST fusion protein was removed by multiple washing with cold phosphate buffer (pH 7.4) containing 1% BSA. Bound ^{125}I-labelled F2-GST was extracted from the wells by incubation with 200 μl of 1% SDS at room temperature for 1 h, and radioactivity was determined using a Beckman model LS 5000 CE scintillation counter. Specific binding was determined by subtracting counts of BSA-coated wells from the total counts of wells containing tubulin. Scatchard analysis was performed as described previously (Ciruela et al., 1995), and the data were analysed for one- or two-site binding as described by Casado et al. (1990).

RESULTS

Identification of intracellular proteins interacting with the C terminus of mGluR1α

As a preliminary step towards identifying brain proteins that interact with the carboxyl-terminal tail of mGluR1α, the fusion protein termed F2-GST, containing the last 86 amino acids of rat mGluR1α, was used to overlay SDS-polyacrylamide gels of soluble extracts of rat brain, following transfer of the proteins to PVDF membranes. Bacterially expressed GST alone was used as a control for the overlay experiments. The GST or F2-GST proteins bound to the blot were then incubated with a specific goat anti-GST antibody and detected by chemiluminescence. The F2-GST protein specifically recognized six proteins in the S_3 fraction (Fig. 1A, lane 2), namely, p240, p203, p89, p55, p48, and p36 (with apparent molecular masses 240, 203, 89, 55, 48, and 36 kDa, respectively). The interaction with protein p69 was considered to be nonspecific because a protein with a similar molecular size was consistently eluted from the control GST column (Fig. 1A, lane 1).

To purify these proteins, we coupled F2-GST and GST to CNBr-activated Sepharose-4B to produce affinity chromatography columns. The soluble extract (S_3) was first passed through the GST-Sepharose-4B column and then through the F2-GST column. After washing the two columns, the interacting proteins were eluted with 0.1 *M* triethylamine (pH 11.5) and analysed by SDS-PAGE followed by Coomassie Blue staining.

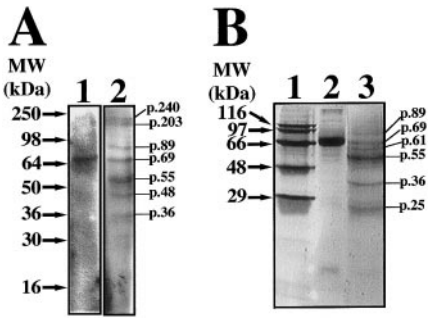

FIG. 1. Identification and affinity purification of proteins interacting with the 86 C-terminal residues of mGluR1α. **A:** Identification of brain proteins interacting with the C terminus of mGluR1α by gel overlay with fusion proteins. Soluble extracts (20 μg; S_3) obtained from rat brain (see Materials and Methods) were analysed on 10% SDS-PAGE gels, and the proteins were transferred to PVDF membranes and then probed with 3 μ*M* GST (lane 1) or F2-GST (lane 2) proteins. The fusion proteins bound to the blot were then incubated with a specific goat anti-GST antibody and developed using chemiluminescence. Similar results were obtained in three different experiments. The positions of the molecular mass markers in kDa are indicated on the left. **B:** Purification of proteins interacting with the C terminus of mGluR1α by affinity chromatography on an F2-GST column. Soluble extracts (S_3; 1.5 mg/ml) obtained from rat brain were applied to a 2-ml GST (lane 2) or F2-GST (lane 3) affinity column overnight at 4°C. The column was washed, and the proteins were eluted as described in Materials and Methods. The freeze-dried eluate was reconstituted in 100 μl of distilled water, and 20 μl of the sample was analysed on a 10% SDS-PAGE gel. The proteins were transferred to PVDF and Coomassie Blue-stained. Lane 1, molecular mass markers.

The eluate from the F2-GST-Sepharose-4B column contained five specific proteins in high yield, namely, p89, p61, p55, p36, and p25 (with apparent molecular masses 89, 61, 55, 36, and 25 kDa, respectively; Fig. 1B, lane 3). A major band, p69, was also eluted from both the GST-Sepharose-4B and the F2-GST columns (Fig. 1B, lane 2). Several of these affinity-purified proteins appeared to correspond in size to those identified from the overlay experiments. Three of these proteins specifically eluted from the F2-GST column, namely, p55, p36, and p25, yielded N-terminal protein sequences. The N-terminal sequence of the p55 protein was identified as MREIVHIQAGQCGNQ, that of the protein p35 was VKVGVNGFGR, and that of p25 as ALD(Y/T)LA-(E/D)AGA(L/E)(N/V)AN. The residues underlined in the latter sequence were not unambiguously determined because either they were obtained in low yield or more than one amino acid was identified in that cycle of the analysis. Analysis of protein databases showed p55 to be β-tubulin and p35 to be glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and no match was found for p25 despite trying different permutations of the identified sequence.

Identification of β-tubulin by immunoblotting

To characterize further the proteins isolated from the affinity columns, different rat brain and column fractions were analysed by SDS-PAGE followed by immunoblotting with different antibodies (Fig. 2). When the blot was probed with a C-terminal anti-mGluR1α antibody (F2-Ab), an immunoreactive band of mGluR1α with an apparent molecular size of 150 kDa was observed in the total brain and synaptosome fractions (Fig. 2, lanes A and B). As expected no mGluR1α could be detected in the soluble S_3 fraction or in the proteins eluted from either the GST or F2-GST-Sepharose-4B columns (Fig. 2, lanes C–E).

Reacting the immunoblot with a monoclonal antibody to β-tubulin yielded an immunoreactive band with the expected apparent molecular size of 55 kDa found in total brain, the synaptosomes, the soluble S_3 fraction, and the proteins eluted from the F2-GST-Sepharose-4B column (Fig. 2, lanes A–C and E). No tubulin immunoreactivity was found in the proteins eluted from the control GST-Sepharose-4B column (Fig. 2, lane D). This confirmed that β-tubulin bound specifically to the carboxy-terminal tail of mGluR1α.

Gephyrin, the protein that acts as a bridge between tubulin and the inhibitory glycine receptor and is implicated in its postsynaptic localization, has been shown to bind tubulin (Kirsch and Betz, 1995). Therefore, the specific F2-GST column eluates were examined for the presence of both gephyrin and PSD95, which has been shown to react with the C-terminal residues of specific NMDA receptor and potassium channel subunits (Hunt et al., 1996; Kim and Sheng, 1996). The anti-gephyrin antibody gave an immunoreactive band of the expected molecular size (93 kDa) in the total brain, synaptosomal, and soluble fractions but not in the specific eluate from the F2-GST column (Fig. 2, lanes A–C). The monoclonal anti-PSD95 yielded an immunoreactive band with the predicted apparent molecular size (93 kDa) in the total brain and synaptosomal fractions and not in the soluble

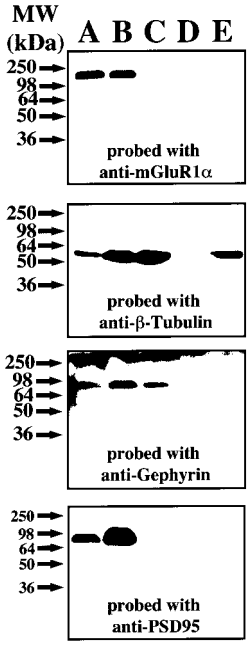

FIG. 2. Immunoblot analysis of the affinity column eluates. Samples (20 μg) of total rat brain (lane A), rat brain synaptosomes (lane B), rat brain soluble fraction (lane C), and eluted proteins from a GST (lane D) or F2-GST (lane E) column (5 μl; see Fig. 1) were analysed on 10% SDS-PAGE gels and transferred to PVDF membranes. Immunoblotting was performed as described in Materials and Methods using the specified antibodies as follows: anti-mGluR1α (F2-Ab; 2–4 μg/ml), monoclonal anti-β-tubulin (1:200), monoclonal anti-gephyrin (1:500), and monoclonal anti-PSD95 (1:500). Immunoreactive bands were detected with swine anti-rabbit (1:5,000) or sheep anti-mouse (1:5,000) secondary antibody conjugated to horseradish peroxidase followed by chemiluminescence detection. Similar results were obtained in three different experiments. The positions of the molecular mass markers in kDa are indicated on the left.

fraction or in the eluates from the F2-GST column (Fig. 2, lanes A and B). Indeed, as previously reported this protein is enriched in the synaptosomal fractions. These results suggest that the F2-GST column is specifically purifying tubulin and that the complex of proteins isolated from the F2-GST column and interacting with the C-terminus of mGluR1α does not contain PSD95 nor does it contain gephyrin, despite the ability of the latter to bind tubulin.

mGluR1α interacts with tubulin in brain

To determine if tubulin interacts with the full mGluR1α in brain, coimmunoprecipitation studies with anti-β-tubulin antibodies were performed on solubilized rat brain extracts containing both membrane and cytosolic proteins. The specifically immunoprecipitated proteins were separated on SDS-polyacrylamide gels, and the proteins were transferred to PVDF membranes. These were then immunoblotted using different antibodies. Immunoprecipitation with an N-terminal mGluR1α antibody resulted in the identification of the 150-kDa receptor when the immunoblot was probed with an mGluR1α-specific serum (Fig. 3, lane A). An immunoreactive band with an identical migration was also seen in the immunoprecipitate from the anti-β-tubulin antibody, an indication that the two proteins are indeed associated in the brain (Fig. 3, lane B). In contrast, the immunoprecipitates performed with either anti-gephyrin or anti-PSD95 failed to yield mGluR1α-immunoreactive material, although both sera did immunoprecipitate their respective proteins (Fig. 3, lanes C and D). The anti-β-tubulin immunoprecipitate did, however, contain gephyrin, indicating that these two proteins do indeed interact, whereas the PSD95 immunoprecipitates contained only PSD95, demonstrating that the interactions observed with the other sera were indeed specific. The heavily immunoreactive bands seen in all the tracks are due to the immunoglobulin heavy chains derived from the precipitating antibodies. The presence of these meant that it

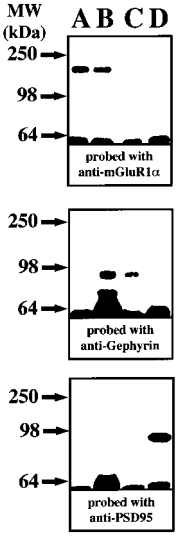

FIG. 3. Coimmunoprecipitation of mGluR1α with anti-β-tubulin. Supernatant S_1 from total rat brain was solubilized in ice-cold lysis buffer as described in Materials and Methods. The solubilized material (1 mg of protein/ml) was immunoprecipitated using specific N-terminal anti-mGluR1 antibody (lane A; 5 μg/ml), monoclonal antibody anti-β-tubulin (lane B; 1:200), monoclonal antibody anti-gephyrin (lane C; 1:200), and monoclonal anti-PSD95 (lane D; 5 μg/ml). After overnight incubation 80 μl of 50% (vol/vol) protein A-Sepharose beads was added, incubated, and pelleted as described in the text. The beads were washed, resuspended in 60 μl of SDS-PAGE sample buffer, and analysed on 7% SDS-polyacrylamide gels. The gels were subsequently immunoblotted using the indicated antisera. The positions of the molecular mass markers in kDa are indicated on the left.

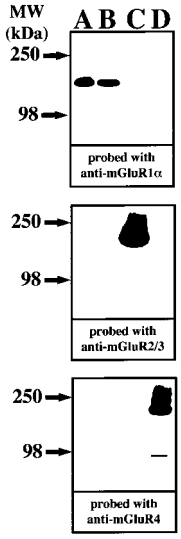

FIG. 4. The coimmunoprecipitation of mGluR1α with tubulin is specific. In a separate experiment supernatant S_1 from total rat brain was solubilized in ice-cold lysis buffer as described in Materials and Methods. The solubilized material (1 mg of protein/ml) was immunoprecipitated using specific N-terminal anti-mGluR1 antibody (lane A; 5 μg/ml), monoclonal antibody anti-β-tubulin (lane B; 1:200), affinity-purified anti-mGluR2/3 (lane C; 15 μg/ml), or affinity-purified anti-mGluR4 (lane D; 10 μg/ml). After overnight incubation 80 μl of 50% (vol/vol) protein A-Sepharose beads was added, incubated, and pelleted as described before. The beads were washed, resuspended in 60 μl of SDS-PAGE sample buffer, and analysed on 7% SDS-polyacrylamide gels. The gels were subsequently immunoblotted using the indicated antisera. The positions of the molecular mass markers in kDa are indicated on the left. Both mGluR2/3 and mGluR3 give major immunoreactive bands at ~180 kDa with minor bands apparent at 90 kDa, which is the predicted molecular size for the protein. This is due to the formation of dimers of these receptors as has been noted previously (Alaluf et al., 1995; Shigemoto et al., 1997). The immunoglobulin heavy chain bands are not visible on this immunoblot because the gel was subjected to electrophoresis for longer than shown in Fig. 3.

was not possible to perform the reciprocal experiment of immunoblotting for tubulin in the immunoprecipitates of mGluR1α, as the tubulin would have migrated very close to these immunoglobulin heavy chains, and this would have obscured any immunoreactivity due to tubulin.

To determine if other members of the mGluR family interact with tubulin, a similar experiment was performed using solubilized rat brain and performing immunoprecipitation with anti-β-tubulin together with specific antibodies to mGluR2/3 and mGluR4. The results showed that both mGluR2/4 and mGluR4 could be found in and immunoprecipitated from the brain extracts, but that these mGluRs could not be coimmunoprecipitated with anti-β-tubulin antibody, although mGluR1α could (Fig. 4).

The interaction of mGluR1α with tubulin is direct

To determine whether the interaction of mGluR1α with tubulin was direct or mediated via another protein, overlay experiments were performed using the F2-GST protein (Fig. 5, lane C) and purified tubulin (Fig. 5, lane A) that had been transferred to PVDF membranes following SDS-polyacrylamide gel separation. The purified tubulin contained both α- and β-tubulin, which migrated as a protein doublet. The results showed that in the lane containing purified tubulin a band that reacted with the F2-GST protein was seen at the expected position in the gel (Fig. 5, lane D), whereas GST alone did not give any reaction (Fig. 5, lane E). These results suggested that mGluR1α interacts directly with tubulin through its carboxy terminus, but the resolution of the overlay experiments was not such as to allow us to determine if the

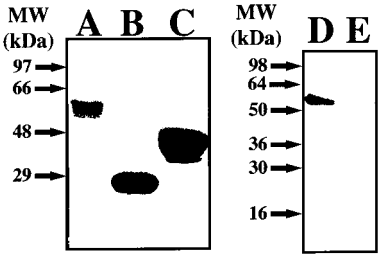

FIG. 5. The interaction of mGluR1α with tubulin is direct. Purified tubulin (lane A; 8 μg), GST (lane B; 30 μg), and F2-GST (lane C; 40 μg) were analysed on a 10% SDS-polyacrylamide gel, and the purity was checked by staining with Coomassie Blue. In parallel, tubulin (8 μg) was electrophoresed and transferred to PVDF membranes. After blocking the membranes they were overlaid with either the F2-GST fusion protein (lane D; 3 μ*M*) or the control GST protein alone (lane E). Immunoreactive bands were detected with anti-GST antibodies and chemiluminescence development. The positions of the molecular mass markers in kDa are indicated on the left.

F2-GST fusion protein was interacting only with β-tubulin.

To confirm that the binding is direct and to determine the affinity of the C-terminal fusion protein for tubulin, a direct binding assay for the two proteins was established using highly purified iodinated F2-GST and tubulin (Fig. 6A). The specificity of the interaction of the two proteins is shown by the fact that only unlabelled F2-GST could compete with the labelled probe, whereas GST alone or a GST fusion protein containing the C-terminal tail of mGluR4 could not do so (Fig. 6B). The latter result confirms the earlier observation that mGluR4 could not be coimmunoprecipitated with anti-β-tubulin antibodies (Fig. 4). Equilibrium binding studies on the binding of F2-GST to tubulin gave saturable binding, which when analysed as described previously (Casado et al., 1990) gave a best fit to a single site with an apparent K_D of 2.3 μ*M* (Fig. 6C). Together these studies show that the fusion protein and presumably the C-terminal tail of mGluR1α can bind directly to tubulin.

DISCUSSION

The results presented here show that the fusion protein containing the last 86 residues of the C-terminal tail of mGluR1α can be used to isolate specifically at least five rat brain proteins, from three of which we have been able to obtain sequence information. Two of these were identified as GAPDH and β-tubulin, respectively, whereas the third yielded a less satisfactory sequence that could not be used to identify the protein. This last protein has not been studied here but remains under investigation.

GAPDH is a well-recognized glycolytic enzyme that has recently been identified as playing diverse roles in cell metabolism, including endocytosis, mRNA regulation, and DNA repair (for review, see Sirover, 1997). Furthermore, the enzyme also interacts directly with several proteins that contain CAG repeats such as Huntingtin (Burke et al., 1966) and with the C-terminal tail of β-amyloid precursor (Schulze et al., 1993). Using the C-terminal tail of the intracellular adhesion molecule-1 as an affinity ligand, much as we used the C-terminal tail of mGluR1α here, Federici et al. (1996) copurified tubulin and GAPDH. Further investigation showed that the

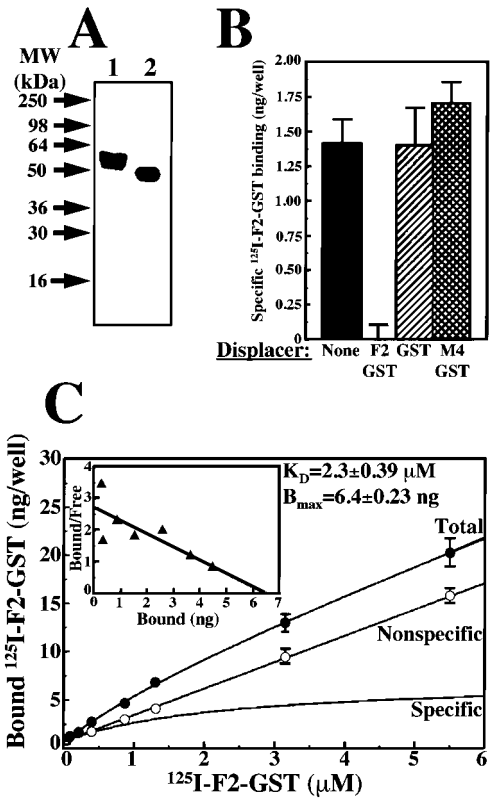

FIG. 6. Determination of the binding affinity of tubulin for F2-GST. **A:** Coomassie Blue-stained gel shows purified tubulin (lane 1). Lane 2 shows an autoradiograph of the radioiodinated F2-GST probe to indicate the purity of the reagents used in the assay. **B:** Specificity of the F2-GST–tubulin interaction under nondenaturing conditions in a solid-phase binding assay. Microtitre wells were coated with tubulin (8 μg per well), and after blocking as described in Materials and Methods, the plates were incubated with 0.75 μ*M* ^{125}I-labelled F2-GST fusion protein in the absence (None) or in the presence of an excess (300-fold) of competing proteins: F2-GST, GST, and M4-GST. Data are mean ± SEM (bars) values of four replicates. Only the F2-GST protein displaced the labelled protein. **C:** Equilibrium binding isotherm of ^{125}I-labelled F2-GST fusion protein to immobilized tubulin. Immobilized tubulin (8 μg) was incubated with different concentrations of ^{125}I-labelled F2-GST (●) in phosphate buffer (pH 7.4) containing 1% BSA overnight at 4°C as described in Materials and Methods. Nonspecific binding (○) was determined by the binding of ^{125}I-labelled F2-GST to 1% BSA-coated wells. The specific binding was calculated by subtracting the nonspecific binding from the total binding. Data are mean ± SEM (bars) values of four replicates. **Inset:** Scatchard plot of the computer-derived specific binding data. The plot was linear (r = 0.93), revealing an apparent single binding site, with a K_D of 2.3 ± 0.39 μ*M* and a B_{max} of 6.4 ± 0.23 ng per tubulin-coated well. Although the data could suggest the presence of a higher-affinity binding site, the goodness of fit to both a single-site and two-site model was analysed as described previously (Casado et al., 1990), and no significant improvement in fit to a single site was obtained when the data were fitted to a two-site model.

tubulin binding of the intracellular adhesion molecule-1 and tubulin was mediated by the C-terminal domain of GAPDH, leading to the suggestion that the GAPDH was acting as an adaptor molecule, linking the intracellular adhesion molecule-1 to the microtubule network (Federici et al., 1996). The direct interaction of the mGluR1α C terminus with tubulin shown here would seem to rule out such a role for GAPDH with mGluR1α. Other studies have also shown that GAPDH can interact with tubulin (Kumagai and Sakai, 1983; Huitorel and Pantaloni, 1985), and it may therefore have been copurified with tubulin in the course of our affinity chromatography using the mGluR1α C-terminal fusion protein. We have therefore not pursued the possibility of the receptor's interaction with GAPDH further.

The direct interaction of mGluR1α with tubulin described here is reminiscent of a similar interaction reported for the α1 subunit of $GABA_A$ receptors (Item and Sieghart, 1994; Kannenberg et al., 1997). This was identified using an approach similar to that described here, although the affinity of the interaction of tubulin with these receptors was not reported. The glycine receptor may also be bound to tubulin, although in this case the interaction is mediated by the protein gephyrin, which has been shown to interact directly with tubulin with a high affinity [K_D = 2.5 n*M* (Kirsch et al., 1991, 1995; Kirsch and Betz, 1995)]. The dissociation constant for the interaction of tubulin with the mGluR1α C-terminal fusion protein is 2.3 μM, which is considerably lower than that of gephyrin for tubulin. However, although the data we obtained were best fitted to a single low-affinity site governing the interaction of tubulin and the C-terminal fusion protein, the presence of a higher-affinity site cannot be excluded. In addition, the actual affinity of the native mGluR1α receptor for tubulin could not be determined in this study. Nevertheless, it is clear from the data presented here that the strength of the interaction between the receptor and tubulin is sufficient to permit the coimmunoprecipitation of the two proteins, which suggests that it could be of physiological significance.

The physiological role for the interaction of mGluR1α with tubulin is not addressed by this study. However, there is clearly evidence for the presence of tubulin in both synaptic membranes (Bhattacharyya and Wolff, 1976; Yan et al., 1996) and PSDs, as, interestingly, is GAPDH (Walsh and Kuruc, 1992). Therefore, there exists the potential for interactions between the receptor and tubulin. The direct interaction of mGluR1α with tubulin could function to stabilize the receptor at a specific location, by interaction with the cytoskeleton. A similar cytoskeletal interaction, although mediated by other proteins, has been suggested to result in the clustering at specific locations of the voltage-dependent sodium channels (Srinivasan et al., 1988), the glycine receptor (Kirsch et al., 1991), and the nicotinic acetylcholine receptor (Hill, 1992). Alternatively, the interaction with tubulin may play a role in the transport of mGluR1α to the synapse.

Tubulin, which itself binds and can hydrolyse GTP (Carlier, 1982), has been shown to interact with the heterotrimeric G proteins $G\alpha_s$, $G\alpha_{i1}$, and $G\alpha_q$ (Wang et al., 1990; Popova et al., 1997), and this interaction can modulate the agonist-mediated responses of the receptors coupled to these G proteins. Indeed, there is evidence that synaptic membrane G proteins are complexed with tubulin in vivo (Yan et al., 1996). Furthermore, anti-tubulin antibodies have been demonstrated to increase the rate of G protein-mediated GTP hydrolysis in response to acetylcholine treatment of rat striatal membranes (Ravindra and Aronstam, 1990). Together these data have led to the suggestion that tubulin modulates receptor responses by interacting with specific G protein α subunits, facilitating GTP transfer, and stabilizing the active Gα conformation (Roychowdhury et al., 1993; Roychowdhury and Raesnick, 1994). The domains responsible for the interaction of G proteins with mGluR1α have been identified as being the second and third intracellular loops of the protein and a short intracellular region of the C terminus, close to the last transmembrane domain (Pin et al., 1994; Francesconi and Duvoisin, 1998; Mary et al., 1998). The C terminus of mGluR1α, although not contributing to G protein binding, may be modulating receptor–G protein interactions by binding tubulin, thereby bringing it into the proximity of the G protein binding domains, where it can act as described above.

The evidence presented here suggests that the mGluR1α receptor C terminus can interact directly with tubulin. It should be noted the methods used here would detect only the most abundant proteins bound to the fusion protein. This could explain why we did not find significant amounts of HOMER in the eluate from our fusion protein column, as the expression of this protein is low in unkindled brain (Brakeman et al., 1997). Given that the fusion protein used in this study represents only 25% of the total C-terminal sequence of this receptor, it is likely that other proteins are going to be complexed with the remainder of the large intracellular domain of this protein. The characterization of these interactions should shed considerable light on the targeting and regulation of this important glutamate receptor.

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