Journal of Neurochemistry, 2001, **76**, 750–757

Metabotropic glutamate receptor type 1α and tubulin assemble into dynamic interacting complexes

Francisco Ciruela and R. A. Jeffrey McIlhinney

Medical Research Council, Anatomical Neuropharmacology Unit, Oxford, UK

Abstract

Metabotropic glutamate receptors (mGlu receptors) are coupled to G-protein second messenger pathways and modulate glutamate neurotransmission in the brain, where they are targeted to specific synaptic locations. Very recently, we identified tubulin as an interacting partner of the $mGlu_{1\alpha}$ receptor in rat brain. Using BHK-570 cells permanently expressing the receptor we have shown that this interaction occurs predominantly with soluble tubulin, following its translocation to the plasma membrane. In addition, treatment of the cells with the agonist quisqualic acid induce tubulin depolimerization and its translocation to the plasma membrane. Immunofluorescence detection of both the receptor and tubulin in agonist-treated cells reveals a disruption of the microtubule network and an increased clustering of the receptor. Collectively these data demonstrate that the $mGlu_{1\alpha}$ receptor interacts with soluble tubulin and that this association can take place at the plasma membrane.

Keywords: $mGlu_{1\alpha}$ receptor, tubulin-interaction-cell surface.
J. Neurochem. (2001) **76**, 750–757.

Glutamate is the major excitatory neurotransmitter in the mammalian brain and acts at multiple receptor types. These receptors can be divided into two groups, namely the ionotropic receptors, which form ion channels, and metabotropic receptors coupled by G-proteins to various second messenger systems (Pin and Duvoisin 1995). Eight members of the mGlu receptor family have been identified and categorized into three subgroups on the basis of their sequence homology, agonist selectivity and signal transduction pathway. Group I contain $mGlu_1$ and $mGlu_5$ subtypes which are coupled to phospholipase C in transfected cells and have quisqualic acid (Quis) as their most potent agonist. Five splice variants of $mGlu_1$ receptor have been described namely $mGlu_{1\alpha}$, $mGlu_{1\beta}$, $mGlu_{1c}$, $mGlu_{1d}$ and $mGlu_{1e}$ receptors (Pin *et al*. 1992; Pin and Duvoisin 1995), all of them differing in the length of the C-terminal tail. The functional significance of the different splice variants has not yet been fully explored. It has been suggested that the C-terminal tail, which is intracellular, might play a role in the subcellular targeting of the receptor (Grandes *et al*. 1994). Recently, we have reported that the C-terminus of $mGlu_{1\alpha}$ receptor interacts with tubulin (Ciruela *et al*. 1999a) and that it can regulate the cell surface expression of the receptor (Ciruela *et al*. 1999b).

Immunocytochemical studies have shown that the different groups of mGlu receptors show differential intraneuronal targeting with the group I mGlu receptors being predominantly postsynaptic whilst the group 2 and group 3 receptors are generally presynaptic (Baude *et al*. 1993; Ohishi *et al*. 1995; Shigemoto *et al*. 1996; Shigemoto *et al*. 1997). The precise synaptic location of $mGlu_{1\alpha}$ receptor appears to be highly ordered, with the protein present in an annulus which surrounds the postsynaptic density (Baude *et al*. 1993; Lujan *et al*. 1996). One explanation for the differential neuronal and synaptic targeting of the different mGlu receptors is that it is directed by interactions of their cytoplasmic C-termini with specific targeting proteins located at the postsynaptic density. Recently a family of proteins which interact with the C-terminal tail of the group I mGlu receptors has been described (Brakeman *et al*. 1997; Kato *et al*. 1998; Tu *et al*.

Received May 5, 2000; revised manuscript received September 8, 2000; accepted September 9, 2000.

Address correspondence and reprint requests to Francisco Ciruela, Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, Marti i Franquès 1, 08028 Barcelona, Spain. E-mail: recep@sun.bq.ub.es

Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; GPCR, G protein-coupled receptor; MAP, microtubule-associated protein; $mGlu_{1\alpha}$ receptor, metabotropic glutamate receptor type 1α; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, phosphoinositide; PVDF, polyvinylidene fluoride; SDS, sodium dodecylsulphate.

1998; Xiao *et al*. 1998). Whilst these proteins may serve to link the mGlu receptors to a variety of signalling proteins they appear to mediate mGlu receptor cytoskeletal protein interactions only indirectly (Tu *et al*. 1999). Interestingly, several cytoskeletal proteins have been found within the postsynaptic density namely tubulin, actin and spectrin, with the former representing up to 14% of its total protein (Kelly and Cotman 1978). The physiological role for the high tubulin content in the postsynaptic density is not fully understood. The direct interaction of tubulin with glutamate receptors (Ciruela *et al*. 1999a; van Rossum *et al*. 1999) 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 of the voltage dependent sodium channels (Srinivasan *et al*. 1988), the glycine receptor (Kirsch *et al*. 1991) and the nicotinic acetylcholine receptor (Hill 1992).

In order to elucidate the role of $mGlu_{1\alpha}$ receptor/tubulin interaction we have studied this interaction in a heterologous dynamic system namely BHK-570 cells permanently transfected with $mGlu_{1\alpha}$ receptor and used co-immunoprecipitation, immunofluorescence and cell surface biotinylation techniques to delineate the conditions in which the interaction takes place.

Materials and methods

Materials

Baby hamster kidney (BHK-570) cells permanently transfected with metabotropic glutamate receptor $mGlu_{1\alpha}$ receptor were kindly provided by Dr E. Mulvihill (Zymogenetics, WA, USA). Tran-[^{35}S]-methionine (> 1000 Ci/mmol) was purchased from ICN (Thame, UK). Dulbecco's modified Eagle's medium (DMEM), Hank's buffered saline solution, methatrexate, trypsin–EDTA, Geneticin G-418 sulphate, colchicine, streptavidin–agarose and protein A-Sepharose were obtained from Sigma (Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK). The western blotting chemiluminescence detection system and the Sulfo-NHS-LC-Biotin reagent were purchased from Pierce (Rockford, IL, USA).

Antibodies

The primary antibodies used for immunolabeling were: anti-β-tubulin monoclonal antibody (Clone TUB 2.1, Sigma-Aldrich Company Ltd), anti-Golgi p58K protein monoclonal antibody (Clone 58K-9, Sigma-Aldrich Company Ltd), affinity purified anti-mGluR1 polyclonal antibody F1-Ab (pan-mGluR1) (Ciruela and McIlhinney 1997) and affinity purified anti-$mGlu_{1\alpha}$ receptor polyclonal antibody F2-Ab (Ciruela *et al*. 1999a). The secondary antibodies used were: horseradish peroxidase (HRP)-conjugate swine anti-rabbit IgG and HRP-conjugate swine antimouse IgG (Dako, Ely, UK); fluorescein (FITC)-conjugate affinity purified donkey anti-mouse IgG and Texas red dye-conjugate affinity purified donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., PA, USA).

Mammalian cell culture and metabolic Tran-^{35}S labelling

BHK-570 cells stably expressing $mGlu_{1\alpha}$ receptor were grown in DMEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 10% (v/v) fetal calf serum (FCS), 0.5 mg/mL G418 sulphate, 1 μM methatrexate at 37°C and in an atmosphere of 5% CO_2. Cells were passaged when 80–90% confluent. Microtubule depolymerization was induced by addition of the alkaloid drug colchicine (10 μM) for the indicated times.

Metabolic labelling of BHK-570 cells permanently transfected with $mGlu_{1\alpha}$ receptor was performed as described previously (Robbins *et al*. 1999). One hour before labelling the DMEM medium was replaced with methionine-free DMEM supplemented with 100 U/mL penicillin-streptomycin. The cells were then incubated with 100 μCi/mL Tran-[^{35}S]-methionine label for 4 h at 37°C. Cells were washed twice with PBS and then lysed with 1 mL of PBS containing 1% Triton 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 for 60 min with 10 μL of preimmune rabbit serum. Then 80 μL of a 50% (v/v) suspension of protein A-Sepharose beads was added, all the mixture was incubated for a further 1–2 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 F1-Ab (5 μg) or TUB 2.1 (2 μL). After overnight incubation 80 μL of protein A-Sepharose was added to each immunoprecipitate and then rotated for 2–3 h as above. The protein A-IgG-receptor immunocomplex was pelleted at 900 *g* in a microfuge for 5 min at 4°C, resuspended, washed twice with 1 mL 1% Triton in PBS, twice with 0.1% Triton in PBS and once with PBS. The pellet was then resuspended in 2 × 50 μL of sample buffer containing 20 mM dithiothreitol (DTT), 4% (w/v) SDS, 20% (v/v) glycerol, 100 mM Tris-HCl (pH 6.8) and a trace of bromophenol blue (SDS/PAGE sample buffer). The immunoprecipitated protein was eluted by incubation at 100°C for 5 min. Eluted proteins were resolved by SDS–polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-polyacrylamide gel electrophoresis was performed on 7–10% gels using a discontinuous Tris-glycine buffer system (Laemmli 1970). SDS-polyacrylamide gels containing ^{35}S were then impregnated with 2,5-diphenyloxazole (PPO) (Bonner and Laskey 1974). The dried gels were then exposed to Kodak X-OMAT film at − 70°C.

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) and monoclonal anti-p58K antibody (1 : 500). Immunoreactive bands were visualized using a 2-h incubation with either swine antirabbit or sheep antimouse antibody conjugated to horseradish peroxidase at room temperature, followed by chemiluminiscence detection.

Biotinylation of cell surface proteins

Biotinylation was performed as described previously (Ciruela *et al*.

1999b) using 50 μg/mL Sulfo-NHS-LC-Biotin (Pierce) in borate buffer for 5 min at room temperature. Biotinylated cell surface proteins were isolated using streptavidin–agarose beads and immunoblotted as described above.

Immunofluorescence

For immunofluorescence staining, BHK-570 cells growing on glass cover-slips were rinsed in PBS, fixed in 4% paraformaldehyde in PBS for 15 min, and washed in PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. Cells were permeabilized with buffer A containing 0.2% Triton X-100 for 5 min. After a 30-min incubation in buffer A containing 1% BSA (buffer B), cells were incubated first with a mixture of antimGlu1α receptor (F2-Ab, 2–4 μg/mL) and anti-β-tubulin monoclonal antibody (Clone TUB 2.1, 1 : 200) in buffer B for 1 h at room temperature, washed, and stained with fluorescein-conjugated donkey anti-rabbit IgG antibody (1/100) or Texas red-conjugated donkey anti-mouse IgG antibody (1/100). Coverslips were rinsed for 30 min in buffer B and mounted with Vectashield immunofluorescence medium (Vector Laboratories, Orton Southgate, Peterborough, UK).

Confocal microscope observations were made with a Leica TCS NT (Leica Lasertechnik GmbH, Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope.

Results

$MGlu_{1\alpha}$ receptor interacts with soluble tubulin in BHK-570 cells

Recently we demonstrated a direct physical interaction between the cytoplasmatic C-terminal tail of the metabotropic glutamate receptor type 1α and tubulin in the rat brain (Ciruela *et al*. 1999a). To address the potential functional significance of this interaction, we have further characterized the $mGlu_{1\alpha}$ receptor/tubulin interaction in an heterologous system, namely BHK-570 cells expressing the $mGlu_{1\alpha}$ receptor. The cells were labelled with Trans-[^{35}S]-label methionine for 4 h at 100 μCi/mL in the absence or presence of 10 μM colchicine, an alkaloid drug that binds to tubulin and inhibits microtubule formation (Perez-Ramirez *et al*. 1996). Following immunoprecipitation of the labelled proteins with the affinity-purified antibody specific for the N-terminal of $mGlu_1$ (F1-Ab), the isolated proteins in noncolchicine treated cells showed a major specific immunoprecipitated protein that migrated with an apparent molecular mass of 150 kDa, equivalent to that of the $mGlu_{1\alpha}$ receptor (Fig. 1). When a similar lysate was immunoprecipitated with the anti-β-tubulin specific antibody (TUB 2.1), a major immunoprecipitated protein with an apparent molecular mass of 55 kDa (equivalent to that of tubulin) was isolated (Fig. 1). However, under these conditions, very low levels of tubulin were found in the specific $mGlu_{1\alpha}$ receptor immunoprecipitate and a barely detectable band of $mGlu_{1\alpha}$ receptor was found in the tubulin immunoprecipitate (Fig. 1). This is in contrast to our previous results which

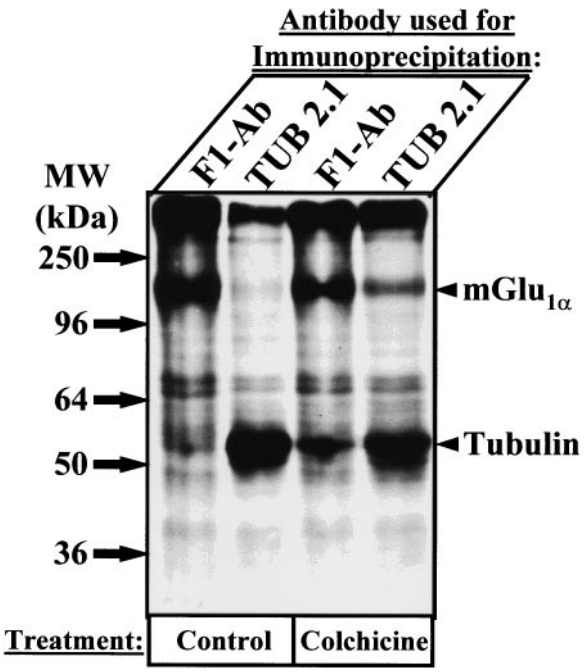

Fig. 1 Characterization of the interaction of $mGlu_{1\alpha}$ receptor and tubulin in BHK-570 cell. Permanently transfected BHK-570 cells with $mGlu_{1\alpha}$ receptor were incubated for 12 h in the presence (colchicine) or absence (control) of 10 μM colchicine and then metabolically labelled with 100μCi/mL Tran-[^{35}S]-methionine for 4 h. Immunoprecipitation was performed as described in the Materials and methods section using either affinity purified anti-$mGluR_1$ antibody (F1-Ab; 5 μg/mL) or anti-β-tubulin monoclonal antibody (clone TUB 2.1, 2 μL). Immunocomplexes were analysed by SDS-PAGE and autoradiographed as described in Materials and methods. The arrows on the right indicate the position of the $mGlu_{1\alpha}$ receptor and tubulin. In this and all subsequent figures the position of the molecular mass markers in kDa are indicated on the left.

showed co-immunoprecipitation of mGlu1α receptor with tubulin from rat brain (Ciruela *et al*. 1999a). However these latter membranes have been shown to contain a large proportion of soluble tubulin (Bhattacharyya and Wolff 1976). This suggested that perhaps the interaction of $mGlu_{1\alpha}$ receptor and tubulin required soluble tubulin. The experiments were therefore repeated using cells that had been treated with colchicine for 12 h to depolymerize the microtubules. The results showed that the levels of tubulin coimmunoprecipitated with the $mGlu_{1\alpha}$ receptor, and those of the $mGlu_{1\alpha}$ receptor co-immunoprecipitated with tubulin, consistently increased following colchicine treatment (Fig. 1), suggesting an increased interaction of the $mGlu_{1\alpha}$ receptor with tubulin dimers or other soluble forms of tubulin.

Cell surface interaction of $mGlu_{1\alpha}$ receptor and tubulin

In order to determine whether the interaction of $mGlu_{1\alpha}$ receptor with tubulin was taking place in the cell surface

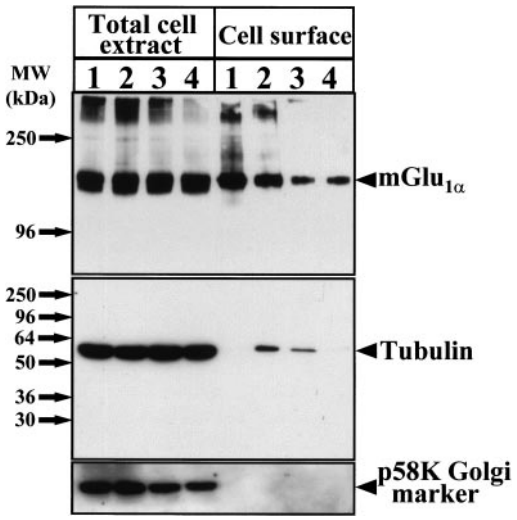

Fig. 2 Cell-surface immunodetection of $mGlu_{1\alpha}$ receptor and β-tubulin in BHK-570 cells. Permanently transfected BHK-570 cells with $mGlu_{1\alpha}$ receptor were incubated for 2 h (lane 2), 6 h (lane 3) and 12 h (lane 4) in the presence or absence (lane 1) of 10 μM colchicine. Crude cell extracts (total cell extract) (40 μg of protein) was analysed by SDS-PAGE and immunoblotted using anti-$mGlu_{1\alpha}$ receptor affinity purified F2 antibody (2–4 μg/mL; upper panel), monoclonal anti-β-tubulin (1 : 200; middle panel) or monoclonal anti-Golgi p58K protein antibody (1 : 100; lower panel). Similarly, permanently transfected BHK-570 cells were cell surface biotinylated, as described in Materials and methods. Biotin-labelled proteins (cell surface) were isolated with streptavidin–agarose beds, analysed by SDS-PAGE and immunoblotted using the same antibodies as above. Immunoreactive bands were detected as described before. The arrows on the right indicate the position of $mGlu_{1\alpha}$ receptor, β-tubulin and p58K Golgi marker.

plasma membrane, proteins were isolated by cell surface protein biotinylation using a membrane-impermeable biotin ester, followed by streptavidin–agarose affinity precipitation of the biotinylated proteins. The results show, under control conditions (Fig. 2, lane 1), that the receptor is present in the cell surface on its own and that tubulin could not be detected in the streptavidin isolates, suggesting again that there was little interaction of $mGlu_{1\alpha}$ receptor and polymerized tubulin (Fig. 2, lane 1). The effect of 10 μM colchicine on the cell surface detection of both $mGlu_{1\alpha}$ receptor and tubulin was then analysed. Treatment of BHK-570 cells permanently transfected with $mGlu_{1\alpha}$ receptor with colchicine reduced the cell surface expression of the receptor in a time-dependent manner, whereas the total amount of receptor in the cells was not affected (Fig. 2, upper panel). This indicates that disruption of the microtubule network had possibly affected the cell surface transport of the receptor. Interestingly, when the streptavidin isolates from colchicine-treated cells were reacted with the anti-β-tubulin antibody it became apparent that tubulin could be detected in cells following a 2-h treatment with colchicine (Fig. 2, middle panel, lane 2). This level of tubulin declined until it could not be detected in cells treated with colchicine for 12 h. This could reflect the loss of cell surface expression of the receptor following prolonged colchicine treatment. Since no p58K protein, a Golgi marker, could be detected in the streptavidin isolates, the biotin ester had not penetrated the cell membrane (Fig. 2, lower panel). These results strongly suggest that soluble tubulin is associated with the receptor at the plasma membrane.

The effect of microtubule depolymerization on the $mGlu_{1\alpha}$ receptor/tubulin interaction by immunofluorescence was then analysed. In control BHK-570 cells permanently transfected with $mGlu_{1\alpha}$ receptor, co-immunostaining of both tubulin and the $mGlu_{1\alpha}$ receptor revealed a little colocalization of both proteins (Fig. 3, panels a, b and c), consistent with the results above (Fig. 1). However when cells were treated with 10 μM colchicine for 4 h and co-immunostained for both $mGlu_{1\alpha}$ receptor and tubulin, a significant disruption of the microtubule network was observed. The tubulin appeared to be redistributed in such a way as to punctate accumulations, some of which colocalized with the $mGlu_{1\alpha}$ receptor (Fig. 3, panels d, e and f). After 12 h of colchicine treatment the cells lost the microtubule network and tubulin became localized to large intracellular accumulations in which the receptor was also found (data not shown). These results are consistent with the hypothesis that the $mGlu_{1\alpha}$ receptor–tubulin interaction requires mainly the soluble form of tubulin.

Agonist-induced cell surface translocation of tubulin

Next, the hypothesis that activated metabotropic glutamate receptor type 1α recruits tubulin to the plasma membrane was tested. BHK-570 permanently expressing $mGlu_{1\alpha}$ receptor were stimulated with the agonist quisqualic acid, and tubulin membrane translocation was assessed by cell surface protein biotinylation and followed by streptavidin–agarose affinity isolation of the membrane proteins. Treatment of cells with 100 μM quisqualic acid did not alter the levels of cell surface expression of the receptor (Fig. 4, upper panel), which is in good agreement with previous results using these cells (Ciruela and McIlhinney 1997) but contrasts with the fast agonist-induced internalization of a fusion protein comprising the $mGlu_{1\alpha}$ receptor and green fluorescent protein (GFP) (Doherty *et al.* 1999). The discrepancy between Doherty's study and the data reported here and previously (Ciruela and McIlhinney 1997) might be due to the different cell lines used (HEK-293 and BHK-570, respectively), since the internalization of G protein-coupled receptors coupled to phospholipase C has previously been shown to be dependent on the cell type (Koenig and Edwardson 1996). When streptavidin-isolated cell surface proteins were probed for

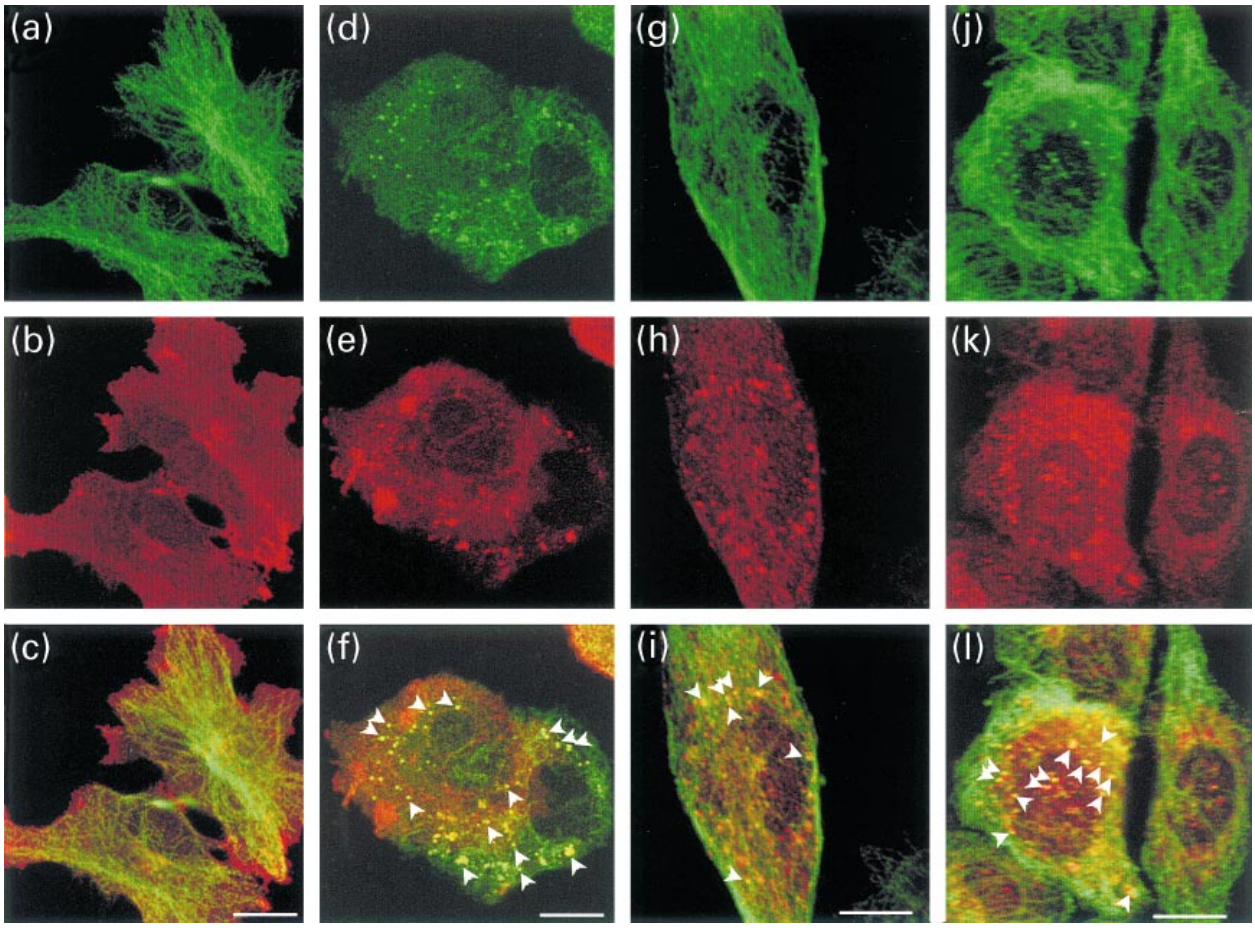

Fig. 3 Immunofluorescent detection of $mGlu_{1\alpha}$ receptor and tubulin in BHK-570 cells. Permanently transfected BHK-570 cells with $mGlu_{1\alpha}$ receptor growing on glass cover-slips (a, b and c) were incubated for 4 h in the presence of 10 μM (d, e and f) of colchicine or 20 min in the presence of 100 μM quisqualic acid (g, h, i, j, k and l) and then processed for immunofluorescence as described in Materials and methods. Immunostaining was performed with a mixture of anti-β-tubulin monoclonal antibody (clone TUB 2.1, 1 : 200) (a, d and g) and anti-$mGlu_{1\alpha}$ receptor (F2-Ab, 2–4 μg/mL) (b, e and h). In panels (j), (k) and (l), after quisqualic acid treatment, cells were washed, fixed and immunostained with anti-$mGlu_1$ receptor (F1-Ab, 2–4 μg/mL) (k). After washing, cells were postfixed with 4% paraformaldehyde in PBS for 5 min, and permeabilized with 0.2% Triton X-100 for 5 min. Cells were then immunostained with anti-β-tubulin monoclonal antibody (clone TUB 2.1, 1 : 200) (j). The bound primary antibody was detected using either Texas red-conjugated donkey antirabbit IgG antibody (1 : 100) or fluorescein-conjugated donkey antimouse (1 : 100). Cells were analysed by double immunofluorescence with a confocal microscopy to detect β-tubulin (green images) and $mGlu_{1\alpha}$ receptor (red images). Superimposition of images (c, f, i and l) reveals tubulin/$mGlu_{1\alpha}$ receptor colocalization in yellow (arrowheads). The scale bar represents 10 μm.

the presence of tubulin, only the cells treated with quisqualic acid showed a small but consistent amount of tubulin associated with the plasma membrane. The quisqualic acid induced plasma membrane translocation of tubulin was rapid (5 min) and declined to background levels after 1 h (Fig. 4, lower panel). A similar rapid and transient agonist induced translocation of tubulin to the plasma membrane has been reported for the muscarinic receptor (Popova and Rasenick 2000).

The effect of quisqualate treatment on the distribution of $mGlu_{1\alpha}$ receptor and tubulin cells was examined by confocal immunofluorescence. Exposure of BHK-570 permanently expressing $mGlu_{1\alpha}$ receptor to 100 μM quisqualic acid during 10 min induced reorganization of $mGlu_{1\alpha}$ receptor in these cells, showing a clustering of the receptor (Fig. 3, panel h). Interestingly, agonist-treated cells also showed a tubulin reorganization which became more punctate with some of the punctae colocalizing with $mGlu_{1\alpha}$ receptor (Fig. 3, panels g, h and i).

Since the cell surface biotinylation data suggested an association of tubulin with $mGlu_{1\alpha}$ receptor in the plasma membrane, we have attempted to confirm this using immunofluorescence. BHK-570 cells permanently expressing $mGlu_{1\alpha}$ receptor were treated with 100 μM quisqualic

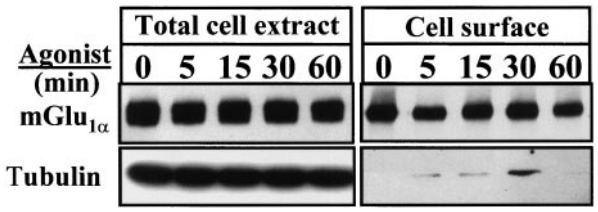

Fig. 4 Cell surface immunodetection of $mGlu_{1\alpha}$ receptor and tubulin in agonist treated BHK-570 cells. Permanently transfected BHK-570 cells with $mGlu_{1\alpha}$ receptor were incubated 5 min, 15 min, 30 min and 1 h in the presence or absence of 100 μM quisqualic acid. Crude cell extracts (total cell extract) (40 μg of protein) was analysed by SDS-PAGE and immunoblotted using antim$Glu_{1\alpha}$ receptor affinity purified F2 antibody (2–4 μg/mL; upper panel), monoclonal anti-β-tubulin (1 : 200; lower panel). Similarly, permanently transfected BHK-570 cells were cell surface biotinylated as described in Materials and methods. Biotin-labelled proteins (cell surface) were isolated with streptavidin–agarose beds, analysed by SDS-PAGE and immunoblotted using the same antibodies as above. Immunoreactive bands were detected as described in Materials and methods.

acid during 10 min, fixed with paraformaldehyde and reacted with the F1-Ab (Fig. 3, panel k). The cells were then washed, postfixed, permeabilized, and incubated with the anti-β-tubulin antibody (Fig. 3, panel j). The results showed clearly that some of the clusters of receptor in the cell surface corresponded precisely with the intracellular accumulations of tubulin, suggesting that the two proteins are indeed closely associated and occur at the plasma membrane (Fig. 3, panel l).

Under these conditions, a change in the cell morphology was observed, with the agonist-treated cells showing a more elongated shape. This is in good agreement with previously reported results (Kubo *et al*. 1998) where the transient transfection and activation of $mGlu_{1\alpha}$ receptor in fibroblasts induced the cells to form a spindle or bar shape.

Discussion

In the brain the processes involved in the generation and maintenance of synapses are critical for neuronal development. Within this processes cytoskeletal dynamics have been found to play a very important role in synaptogenesis and synaptic plasticity, especially following excitatory neurotransmission (Fischer *et al*. 1998).

Our data reveal an interaction between soluble forms of tubulin and the metabotropic glutamate receptor type 1α. This interaction takes place at the plasma membrane level and, more interestingly, agonist activation of the receptor results in a translocation of tubulin to the plasma membrane. Thus, the $mGlu_{1\alpha}$ receptor/tubulin interaction may represent a novel link between cell signalling and cytoskeletal molecules. Indeed, many studies have revealed functional relationships between tubulin and various cellular signalling molecules. For example, tyrosine kinases such as the insulin receptor and c-Src, as well as second messenger-responsive kinases, such as the cAMP-, Ca^{2+}/calmodulin-dependent kinases and G protein-coupled receptor kinases, can regulate microtubule function and/or assembly through phosphorylation (MacRae 1997). Moreover, a series of recent studies have demonstrated direct binding interactions between G_{α} and $G_{\beta\gamma}$ subunits and tubulin (Wang *et al*. 1990; Roychowdhury and Rasenick 1997) suggesting new modes of regulation for microtubule assembly. Other studies provide examples of tubulin or microtubules regulating either the activity or the localization of signalling molecules such as the A1 adenosine (Saunders and Limbird 1997) and γ-aminobutyric acid receptors (Whatley *et al*. 1994), phospholipase C-β1 (Popova *et al*. 1997), and Ki-ras (Thissen *et al*. 1997).

Agonist activation of $mGlu_{1\alpha}$ receptor induces translocation of soluble tubulin to the plasma membrane, probably by depolymerization of existing microtubules. The binding of MAPs to tubulin promotes tubulin polymerization and the formation of microtubules, and Huang and Hampson (2000) have recently demonstrated that coexpression of $mGlu_{1\alpha}$ receptor with MAP2c in MG-1 insect cells causes an inhibition of the growth of processes that is induced by the expression of MAP2c alone, a process mediated by phosphoinositides (PIs). Activation of $mGlu_{1\alpha}$ receptor may locally raise the levels of PIs that will bind to MAPs and thereby inhibit their ability to promote tubulin polymerization (Huang and Hampson 2000). On the other hand, the ability of tubulin to interact with membranes has been shown to be regulated by the phosphorylation level of tubulin (Hargreaves *et al*. 1986); tubulin phosphorylated on the C-terminal tyrosine by the insulin receptor fails to polymerize into microtubules (Wandosell *et al*. 1987). Indeed, the metabotropic glutamate receptor type 1α has been shown to activate tyrosine kinases (Umemori *et al*. 1997; Ferraguti *et al*. 1999) which could provide a mechanism for tubulin phosphorylation and its subsequent plasma membrane translocation.

The results presented here show that activation of $mGlu_{1\alpha}$ receptor can cause alterations in the microtubules of cells, and that depolymerized tubulin can associate with the receptor at the cell surface. This suggests that in synapses, activation of the receptor may result in similar changes with important consequences for events dependent on microtubules such endocytosis, exocytosis, maintenance of cell shape, vesicle trafficking and cellular transport (Mandelkow and Mandelkow 1992). The results of $mGlu_{1\alpha}$ receptor activation on the microtubule network may also have consequences for synaptic remodelling and synaptic plasticity.

Acknowledgements

FC was awarded a fellowship from The Wellcome Trust and he is currently holding a research contract signed with the Catalan

Commission for Research and Technological Innovation (CIRIT).

References

Baude A., Nusser Z., Roberts J. D. B., Mulvihill E., McIlhinney R. A. J. and Somogyi P. (1993) The metabotropic glutamate receptor (mGluR1α) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron* **11**, 771–787.

Bhattacharyya B. and Wolff J. (1976) Polymerization of membrane tubulin. *Nature* **264**, 576–578.

Bonner W. M. and Laskey R. A. (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83–88.

Brakeman P. R., Lanahan A. A., O'Brien R., Roche K., Barnes C. A., Huganir R. L. and Worley P. F. (1997) Homer: a protein that selectively binds metabotropic glutamate receptors. *Nature* **386**, 284–288.

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

Ciruela F., Robbins M. J., Willis A. C. and McIlhinney R. A. J. (1999a) Interactions of the C-terminus of metabotropic glutamate receptor type 1α with rat brain proteins: Evidence for a direct interaction with tubulin. *J. Neurochem.* **72**, 346–354.

Ciruela F., Soloviev M. M. and McIlhinney R. A. J. (1999b) Cell surface expression of the metabotropic glutamate receptor type 1α is regulated by the C-terminal tail. *FEBS Lett.* **448**, 91–94.

Doherty A. J., Coutinho V., Collingridge G. L. and Henley J. M. (1999) Rapid internalisation and surface expression of a functional, fluorescently tagged G-protein-coupled glutamate receptor. *Biochem. J.* **341**, 415–422.

Ferraguti F., Baldani-Guerra B., Corsi M., Nakanishi S. and Corti C. (1999) Activation of extracellular signal-regulated kinase 2 by metabotropic glutamate receptors. *Eur. J. Neurosci.* **11**, 2073–2082.

Fischer M., Kaech S., Knutti D. and Matus A. (1998) Rapid actin-based plasticity in dendritic spines. *Neuron* **20**, 847–854.

Grandes P., Mateos J. M., Ruegg D., Kuhn R. and Knopfel T. (1994) Differential cellular localization of three splice variants of the mGluR1 metabotropic glutamate receptor in rat cerebellum. *Neuroreport* **5**, 2249–2252.

Hargreaves A. J., Wandosell F. and Avila J. (1986) Phosphorylation of tubulin enhances its interaction with membranes. *Nature* **323**, 827–828.

Hill J. A. Jr (1992) Nicotinic receptor-associated 43K protein and progressive stabilization of the postsynaptic membrane. *Mol. Neurobiol.* **6**, 1–17.

Huang X. P. and Hampson D. R. (2000) Inhibition of microtubule formation by metabotropic glutamate receptors. *J. Neurochem.* **74**, 104–113.

Kato A., Ozawa F., Saitoh Y., Fukazawa Y., Sugiyama H. and Inokuchi K. (1998) Novel members of the Vesl/Homer family of PDZ proteins that bind metabotropic glutamate receptors. *J. Biol. Chem.* **273**, 23969–23975.

Kelly P. T. and Cotman C. W. (1978) Synaptic proteins. Characterization of tubulin and actin and identification of a distinct postsynaptic density polypeptide. *J. Cell Biol.* **79**, 173–183.

Kirsch J., Langosch D., Prior P., Littauer U. Z., Schmitt B. and Betz H. (1991) The 93kDa glycine receptor-associated protein binds to tubulin. *J. Biol. Chem.* **266**, 22242–22245.

Koenig J. A. and Edwardson J. M. (1996) Intracellular trafficking of the muscarinic acetylcholine receptor: importance of subtype and cell type. *Mol. Pharmacol.* **49**, 351–359.

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.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lujan R., Nusser Z., Roberts J. D. B., Shigemoto R. and Somogyi P. (1996) Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the hippocampus. *Eur. J. Neuroscience* **8**, 1488–1500.

MacRae T. H. (1997) Tubulin post-translational modifications – enzymes and their mechanisms of action. *Eur. J. Biochem.* **244**, 265–278.

Mandelkow E. M. and Mandelkow E. (1992) Microtubule oscillations. *Cell Motil. Cytoskeleton* **22**, 235–244.

Ohishi H., Nomura S., Ding Y.-Q., Shigemoto R., Wada E., Kinoshita A., Li J.-L., Neki A., Nakanishi S. and Mizumo N. (1995) Presynaptic localization of a metabotropic glutamate receptor, mGluR7, in the primary afferent neurons: and immunohistochemical study in the rat. *Neurosci. Lett.* **202**, 85–88.

Perez-Ramirez B., Andreu J. M., Gorbunoff M. J. and Timasheff S. N. (1996) Stoichiometric and substoichiometric inhibition of tubulin self-assembly by colchicine analogues. *Biochemistry* **35**, 3277–3285.

Pin J.-P. and Duvoisin R. (1995) The metabotropic glutamate receptors: Structure and functions. *Neuropharmacology* **34**, 1–26.

Pin J.-P., Waeber C., Prezeau L., Bockaert J. and Heinemann. S. F. (1992) Alternative splicing generates metabotropic glutamate receptors inducing different patterns of calcium release in Xenopus oocytes. *Proc. Natl Acad. Sci. USA* **89**, 10331–10335.

Popova J. S., Garrison J. G., Rhee S. G. and Raesnick M. M. (1997) Tubulin, Gq, and phosphatidylinositol 4,5-bisphosphate interact to regulate phospholipase Cβ1 signaling. *J. Biol. Chem.* **272**, 6760–6765.

Popova J. S. and Rasenick M. M. (2000) Muscarinic receptor activation promotes the membrane association of tubulin for the regulation of Gq-mediated phospholipase $C\beta_1$ signaling. *J. Neurosci.* **20**, 2774–2782.

Robbins M. J., Ciruela F., Rhodes A. and McIlhinney R. A. J. (1999) Characterization of the dimerization of the metabotropic glutamate receptor mGluR1α. *J. Neurochem.* **72**, 2539–2547.

van Rossum D., Kuhse J. and Betz H. (1999) Dynamic interaction between soluble tubulin and C-terminal domains of *N*-methyl-D-aspartate receptor subunits. *J. Neurochem.* **72**, 962–973.

Roychowdhury S. and Rasenick M. M. (1997) G protein beta1gamma2 subunits promote microtubule assembly. *J. Biol. Chem.* **172**, 31576–31581.

Saunders C. and Limbird L. E. (1997) Disruption of microtubules reveals two independent apical targeting mechanisms for G-protein-coupled receptors in polarized renal epithelial cells. *J. Biol. Chem.* **272**, 19035–19045.

Shigemoto R., Kulik A., Roberts J. D. B., Ohishi H., Nusser Z., Kaneko T. and Somogyi P. (1996) Target-cell-specific concentration of a metabotropic glutamate receptorin the presynaptic active zone. *Nature* **381**, 523–525.10.

Shigemoto R., Kinoshita A., Wada E., Nomura S., Ohishi H., Takada M., Flor P. J., Neki A., Abe T., Nakanishi S. and Mizuno N. (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J. Neurosci.* **17**, 7503–7522.

Srinivasan Y., Elmer L., Davis J., Bennett V. and Angelides K. (1988) Ankyrin and spectrin associate with voltage-dependent sodium channels in brain. *Nature* **333**, 177–180.

Thissen J. A., Gross J. M., Subramanian K., Meyer T. and Casey P. J. (1997) Prenylation–dependent association of Ki-Ras with

microtubules. Evidence for a role in subcellular trafficking. *J. Biol. Chem.* **272**, 30362–30370.

Tu J. C., Xiao B., Yuan J. P., Lanahan A. A., Leoffert K., Li M., Linden D. J. and Worley P. F. (1998) Homer binds a novel proline rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron* **21**, 717–726.

Tu J. C., Xiao B., Naisbitt S., Yuan J. P., Petralia R. S., Brakeman P., Doan A., Aakalu V. K., Lanahan A. A., Sheng M. and Worley P. F. (1999) Coupling of mGluR/Homer and PSD-95 complexes by the shank family of postsynaptic density proteins. *Neuron* **23**, 583–592.

Umemori H., Inoue T., Kume S., Sekiyama N., Nagao M., Itoh H., Nakanishi S., Mikoshiba K. and Yamamoto T. (1997) Activation of the G protein Gq/11 through tyrosine phosphorylation of the alpha subunit. *Science* **276**, 1878–1881.

Wandosell F., Serrano L. and Avila J. (1987) Phosphorylation of alpha-tubulin carboxyl-terminal tyrosine prevents its incorporation into microtubules. *J. Biol. Chem.* **262**, 8268–8273.

Wang N., Yan K. and Rasenick M. M. (1990) Tubulin binds specifically to the signal-transducing proteins, Gs alpha and Gi alpha 1. *J. Biol. Chem.* **265**, 1239–1242.

Whatley V. J., Mihic S. J., Allan A. M., McQuilkin S. J. and Harris R. A. (1994) Gamma-aminobutyric acidA receptor function is inhibited by microtubule depolymerization. *J. Biol. Chem.* **269**, 19546–19552.

Xiao B., Tu J. C., Petralia R. S., Yuan J. P., Doan A., Breder C. D., Ruggiero A., Lanahan A. A., Wenthold R. J. and Worley P. F. (1998) Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of Homer-related synaptic proteins. *Neuron* **21**, 707–716.