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Metabotropic Glutamate 1α and Adenosine A1 Receptors Assemble into Functionally Interacting Complexes*

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Recently, evidence has emerged that seven transmembrane G protein-coupled receptors may be present as homo- and heteromers in the plasma membrane. Here we describe a new molecular and functional interaction between two functionally unrelated types of G protein-coupled receptors, namely the metabotropic glutamate type 1α ($mGlu_{1\alpha}$ receptor) and the adenosine A1 receptors in cerebellum, primary cortical neurons, and heterologous transfected cells. Co-immunoprecipitation experiments showed a close and subtype-specific interaction between $mGlu_{1\alpha}$ and A1 receptors in both rat cerebellar synaptosomes and co-transfected HEK-293 cells. By using transiently transfected HEK-293 cells a synergy between $mGlu_{1\alpha}$ and A1 receptors in receptor-evoked $[Ca^{2+}]_i$ signaling has been shown. In primary cultures of cortical neurons we observed a high degree of co-localization of the two receptors, and excitotoxicity experiments in these cultures also indicate that $mGlu_{1\alpha}$ and A1 receptors are functionally related. Our results provide a molecular basis for adenosine/glutamate receptors cross-talk and open new perspectives for the development of novel agents to treat neuropsychiatric disorders in which abnormal glutamatergic neurotransmission is involved.

Glutamate is the major excitatory neurotransmitter in the central nervous system (1), and its function through ionotropic and metabotropic (mGlu)[1] glutamate receptors can be modulated by other neurotransmitters/neuromodulators (2). 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 contains $mGlu_1$ and $mGlu_5$ subtypes, which are coupled to phospholipase C in transfected cells, and have quisqualic acid as their most potent agonist. Five splice variants of mGlu1 receptor have been described, $mGlu_{1\alpha}$, $mGlu_{1\beta}$, $mGlu_{1c}$, $mGlu_{1d}$, and $mGlu_{1e}$ receptors (3, 4), all of them differing in the length of their 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 (5). Recently, we have reported that the C terminus of $mGlu_{1\alpha}$ receptor interacts with tubulin (6) and that it can regulate the cell surface expression of the receptor (7) and its plasma membrane anchoring (8, 9).

Adenosine is an important neuromodulator implicated in a variety of brain activities, particularly those related to sleep and ischemic-hypoxic episodes (10). This ubiquitous nucleoside exerts its actions via specific receptors, four of which (A1, A2A, A2B, and A3) have been cloned (11). The A1R is functionally coupled to members of the pertussis toxin-sensitive family of G proteins (G_{i1}, G_{i2}, G_{i3}, and G_o), and its activation regulates several membrane and intracellular proteins such as adenylate cyclase, Ca^{2+} channels, K^+ channels, and phospholipase C (11). Of the multiple neurophysiological actions of adenosine, inhibition of glutamate neurotransmission has been observed in several brain regions (12) and is probably a result of the inhibition of presynaptic calcium influx (13). Apart from this inhibitory effect, there is some evidence documenting functional interactions between adenosine and glutamate receptors in the central nervous system. Of particular interest are reports of group I mGlu receptors signaling being enhanced by group II mGlu receptors in hippocampal and cerebrocortical slices (14–16) and by adenosine A1 receptors in cultured hippocampal type 1 astrocytes (17). Very recently, Toms and Roberts (18) have described that type 2 astrocytes contain group I mGlu receptors coupled to $[Ca^{2+}]_i$ signaling and that co-activation of adenosine A1 receptors enhances group I mGlu-evoked $[Ca^{2+}]_i$ responses in these cells via $G_{i/o}$ G protein-mediated mechanism. Despite these observations, no clear molecular mechanism of this interaction between glutamate and adenosine receptors has been provided yet.

Here we report a molecular interaction between metabotropic glutamate receptor type 1α and the adenosine A1 receptor, two members of different GPCR families. This interaction suggests that both receptors may form part of a signaling complex *in vivo* that could play a critical role in fine-tuning neurotransmission at glutamatergic synapses.

EXPERIMENTAL PROCEDURES

Cell Culture, Generation of $mGlu_1$ Truncated Mutant, and Transfection—Human embryonic kidney cells, HEK-293, were grown as described (9). Rat $mGlu_1$ receptor was truncated after amino acid position 885 (see Fig. 4). A stop codon was introduced into the coding sequence of the FLAG epitope containing $mGlu_{1\alpha}$ receptor cDNA by polymerase chain reaction (8). Forward primer was MGR1-F7 (5′-GGCCCTGGGGTGCATGTTTACTC-3′, position 2833–2855 of the $mGlu_1$ receptor cDNA

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[1] The abbreviations used are: mGlu, metabotropic glutamate; GPCR, G protein-coupled receptors; A1R, adenosine A1 receptors; NMDA, *N*-methyl-D-aspartate; R-PIA,(*R*)-phenylisopropyladenosine; Ab, antibody; GABAa, γ-aminobutyric acid A; PAGE, polyacrylamide gel electrophoresis.

(GenBank™ accession number X57569)), and reverse primer was MGR1-R22 (5′-CGTCTCGAGTT**TAA**TTCCCTGCCCCGGGCTTCTTT-3′, position 3026 to 2992, but also encoded *Xho*I restriction site (underlined) and a stop codon (shown in bold)). The *Bsp*EI-*Xho*I fragment of the amplified cDNA sequence, which contained a stop codon, was used to substitute corresponding fragment of rat $mGlu_{1\alpha}$ receptor cDNA in pcDNA3 vector thus resulting in $mGlu_1$-M7 construct (see Fig. 4). The truncation introduced was confirmed by DNA sequencing.

For the transient expression of $mGlu_{1\alpha}$, $mGlu_{1\beta}$, $mGlu_1$-M7, and/or A1 receptors, cells were transiently transfected with 10 μg of cDNA encoding the rat metabotropic glutamate receptors and/or rat adenosine A1 receptor (ratio 1:1; pcDNA containing LacZ reporter was used to equilibrate the amount of total DNA) by calcium phosphate precipitation (19). The cells were used for experimentation at either 24 or 48 h after transfection. Cells were grown in glutamate-free medium (ICN, Basingstoke, UK) in the absence of both glutamine and glutamic acid for 3 h before their use.

Primary Cultures and N-Methyl-D-Aspartate (NMDA)-induced Neurotoxicity—Cortical hemispheres from E16 rat embryos were dissected, and primary cultures of rat cortical neurons were prepared as described previously (9) and used after 14–21 days *in vitro*. To determine the NMDA-mediated neurotoxicity, the culture-conditioned medium was collected, and the cortical neurons were washed once with serum-free B27-supplemented Neurobasal medium (Life Technologies, Inc.) containing 50 μg/ml gentamicin (Sigma) (BNG medium) and preincubated with or without 30 μM NMDA (Tocris, Bristol, UK) for 10 min at 37 °C, as described previously (20). Quisqualic acid (100 μM) (Tocris, Bristol, UK) and/or (*R*)-phenylisopropyladenosine (R-PIA) (100 nM) (Sigma) were transiently applied for 1 min during the 5 min prior to the addition of NMDA (20) and then added together with the NMDA. Neurons, after being washed with the BNG medium, were returned to the culture-conditioned medium and further incubated at 37 °C for 24 h. After the NMDA pulse (24 h), neurons were stained with propidium iodide (80 μg/ml) for 5 min and examined immediately with a standard epi-illumination fluorescence microscope. Neuronal injury was determined by the ability of propidium iodide (Sigma) to penetrate and interact with DNA in damaged neurons, yielding red fluorescence.

Immunostaining—For immunohistochemistry, rat cerebellum was embedded in OCT and frozen in liquid nitrogen-cooled isopentane. Eight micrometer sections were cut on a cryostat cooled to −18 °C. Sections were collected onto SuperFrost Plus (BDH, Darmstadt, Germany) slides, air-dried, and stored at −70 °C. Sections were blocked for 30 min in 10% donkey serum in Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-HCl, pH 7.5). Slides were incubated with affinity-purified anti-$mGlu_{1\alpha}$ receptor (F2-Ab, 2–4 μg/ml) (21) and affinity-purified anti-A1R (PC21, 5–10 μg/ml) (22) for 1 h at room temperature and then washed twice for 5 min in TBS. Fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson ImmunoReserach Laboratories Inc., West Grove, PA) was applied in TBS at a dilution of 1:100. Section were rinsed and mounted with Vectashield immunofluorescence medium (Vector Laboratories, Orton Southgate, UK).

For immunocytochemistry, HEK-293 cells transiently transfected (see above) and primary cultures of rat cortical neurons were fixed in 4% paraformaldehyde for 15 min and washed with phosphate-buffered saline containing 20 mM glycine (buffer A) to quench the aldehyde groups. Where indicated, cells were permeabilized with buffer A containing 0.2% Triton X-100 for 5 min. Cells were labeled for 1 h at room temperature with the indicated primary antibody, washed, and stained with fluorescein-conjugated donkey anti-rabbit IgG antibody (1/100) and Texas Red-conjugated donkey anti-mouse IgG antibody (1/100). Coverslips were rinsed for 30 min in buffer B and mounted with Vectashield immunofluorescence. 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.

Immunoprecipitation—Rat cerebellum synaptosomes (6) or transfected HEK cells were solubilized in ice-cold lysis buffer (phosphate-buffered saline, pH 7.4, containing 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholic acid, and 0.1% (w/v) 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 as described before (6) using the anti-A1R antibody (PC21-Ab) or anti-FLAG monoclonal antibody (Sigma, Clone M2; 10 μg/ml). The immune complexes were dissociated in SDS-PAGE sample buffer by heating to 100 °C for 5 min and resolved by SDS-polyacrylamide gel electrophoresis in 7% gels (23). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Watford, UK) using a semi-dry transfer system and immunoblotted using the primary antibodies indicated in the figure legends and horseradish-peroxidase-conjugated swine anti-rabbit IgG (Dako, Ely, UK) as a secondary antibody. The immunoreactive bands were developed with the enhanced chemiluminescence detection kit (Pierce), as described previously (6).

Ligand Binding Experiments—Membrane suspensions from transiently transfected HEK cells were obtained according to Casadó *et al.* (24). For competition experiments to determine the K_D values, 30 nM [^{3}H]quisqualic acid (Amersham Pharmacia Biotech) or 20 nM [^{3}H]R-PIA (Amersham Pharmacia Biotech) binding to membrane suspensions of HEK cells transiently expressing $mGlu_{1\alpha}$–FLAG receptor or A1R (0.5 mg protein/ml) were carried out at 25 °C in 50 mM Tris-HCl buffer, pH 7.4, in the absence or presence of increasing amounts of quisqualic acid or R-PIA, as described previously (24). After 2 h of radioligand incubation, free and membrane-bound radioligand were separated by rapid filtration in a Brandel cell harvester through Whatman GF/C filters. Filters were transferred to scintillation vials containing 10 ml of Formula 989 (PerkinElmer Life Sciences). Radioactivity was counted using a Packard 1600 TRI-CARB scintillation counter with 50% of efficiency. Competition data were fitted using a non-linear regression program as described previously (24, 25).

To determine the density of expressed receptors in HEK cells transiently transfected with A1R, $mGlu_{1\alpha}$-FLAG or A1R plus $mGlu_{1\alpha}$–FLAG, the binding of 70 nM [^{3}H]quisqualic and 15 nM [^{3}H]R-PIA was performed as described above, and the B_{max} was deduced taking into account the K_D values as described previously (24).

Calcium Determination—Transiently transfected HEK cells (10^6 cell/ml) were loaded with 5 μM Fura-2/AM for 30 min at 37 °C. Cells were washed and subsequently incubated in HBSS containing 0.2 units/ml adenosine deaminase. Calcium peak induction was achieved by the addition of R-PIA or quisqualic acid. Intracellular calcium was determined at 37 °C in a dual-wavelength Shimadzu RF-5000 spectrofluorophotometer (Shimadzu Europe, Duisberg, Germany) by using the excitation wavelength ratio of 334/366 nm with emission cut-off at 500 nm. Free calcium concentration was calculated as described previously (26).

RESULTS

Interaction of $mGlu_{1\alpha}$ and A1 Receptors in Rat Cerebellum—Immunohistochemical studies showed that mGlu1α receptors are present in cerebellum, and its expression is mainly restricted to the cell body and the dendritic tree of Purkinje cells and basket cells located in the molecular layer (Fig. 1*a*). This is in agreement with the previously described location of these receptors in an annulus, which surrounds the post-synaptic density (27). The A1R had a more ubiquitous distribution in cerebellum being expressed in Purkinje cells and basket cells, as $mGlu_{1\alpha}$ receptor, and also in granule cells (Fig. 1*a*). This fits with the localization of adenosine A1 receptors both presynaptically and postsynaptically (28). In addition, metabotropic glutamate receptor type 1α and adenosine A1 receptors showed a similar distribution in human cerebral cortex being expressed in large pyramidal cells located at layers V and II/III (data not shown).

The *in vivo* co-distribution of $mGlu_{1\alpha}$ and A1 receptors in some cerebellar neurons (Fig. 1*a*) suggests a potential interaction between both receptors at precise brain areas. The existence of $mGlu_{1\alpha}$/A1 receptors' heteromers was assayed by co-immunoprecipitation experiments using a soluble extract from rat cerebellum synaptosomes that had been shown by Western blotting to contain both adenosine A1 (Fig. 1*b, lane 1*) and $mGlu_{1\alpha}$ receptors (Fig. 1*b, lane 2*). When this soluble extract was immunoblotted using a specific antibody against A1 receptor (PC21-Ab), two bands of around 39 and 74 kDa of molecular size were shown (Fig. 1*b, lane 3*); these bands, which are glycosylated proteins (29), have been demonstrated previously to correspond to the adenosine A1 receptor monomer and dimer, respectively (22). On the other hand, a specific antibody against the $mGlu_{1\alpha}$ receptor (F2-Ab) immunoblotted in the cerebellum synaptosomes extract a band with apparent molecular size of 150 kDa that corresponds to the $mGlu_{1\alpha}$ receptor (Fig. 1*b, lane 4*), which is a glycosylated protein as it has been described previously (21, 30). From this extract, the antibody against A1R (PC11-Ab) immunoprecipitated a band of 150 kDa

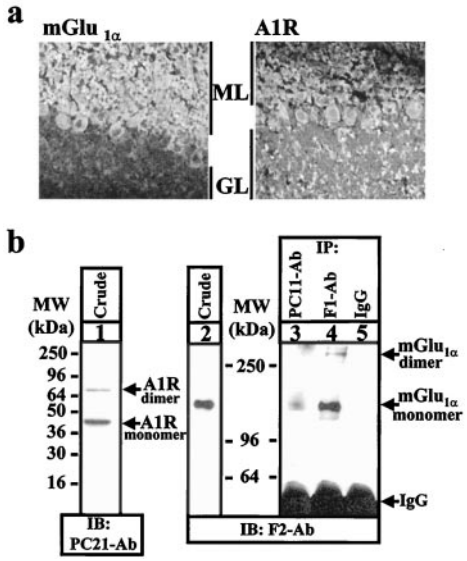

FIG. 1. **Interaction of $mGlu_{1\alpha}$ and A1 receptors in rat cerebellum.** *a,* 8-μm cryosections from rat cerebellum were stained with anti-$mGlu_{1\alpha}$ receptor antibody (F2-Ab; 4 μg/ml) and anti-A1R antibody (PC21-Ab; 10 μg/ml). F2-Ab immunoreactivity is confined mainly to the molecular layer (*ML*) and it stains Purkinje cells and basket cells. PC21-Ab stains Purkinje cells and basket cells in the molecular layer as well as granule cells in the granular layer (*GL*). *b,* co-immunoprecipitation of $mGlu_{1\alpha}$ and A1 receptors from extracts of rat cerebellum synaptosomes. Extracts and immunoprecipitates (*IP*) (see "Experimental Procedures") were analyzed by SDS-PAGE and immunoblotted (*IB*) using anti- $mGlu_{1\alpha}$ receptor antibodies (F2-Ab; 5 μg/ml) or anti-A1R antibodies (PC11-Ab; 10 μg/ml). Immunoreactive bands were detected with swine anti-rabbit (1:5000) secondary antibody conjugated to horseradish peroxidase followed by chemiluminescence detection. IgG indicates the position of the immunoglobulins used in the immunoprecipitation.

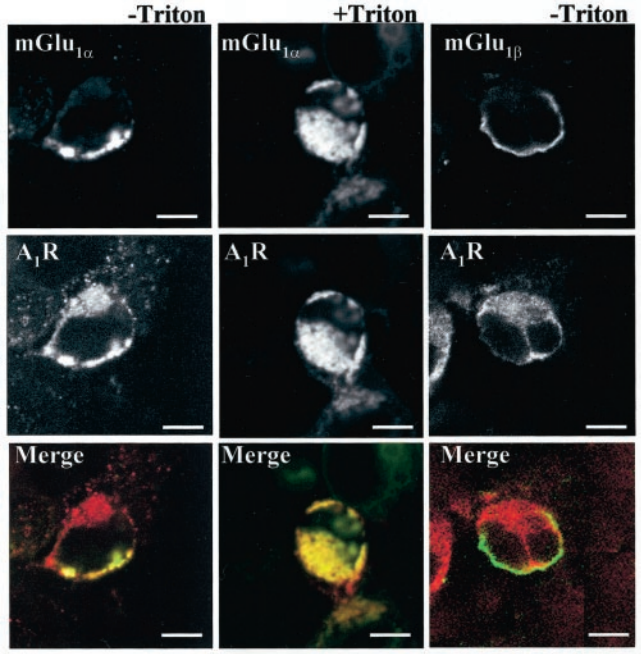

FIG. 2. **Immunofluorescence localization of $mGlu_{1\alpha}$, $mGlu_{1\beta}$, and A1 receptors in HEK-293 cells.** Cells were transiently transfected with cDNAs encoding for $mGlu_{1\alpha}$−FLAG or $mGlu_{1\beta}$−FLAG and A1R. After 48 h cells were washed, fixed (−*Triton*), and/or permeabilized (+*Triton*) and processed for immunostaining with anti-FLAG monoclonal antibody (Sigma, Clone M2; 10 μg/ml) and anti-A1R affinity-purified antibody (PC21-Ab, 5 μg/ml). The bound primary antibodies were detected using either fluorescein-conjugated donkey anti-mouse IgG antibody (1/50) or Texas Red-conjugated donkey anti-rabbit (1/50). Cells were analyzed by double immunofluorescence with a confocal microscopy. Superimposition of images reveals $mGlu_{1\alpha}$ (*green*) and A1 (*red*) receptors co-localization in *yellow*. The images show a single horizontal section of representative cells. *Scale bar*, 10 μm.

that was detected by the F2-Ab (Fig. 1*b*). This band was also immunoprecipitated using a different antibody against $mGlu_1$ receptor (F1-Ab) but was not present in immunoprecipitates generated with an irrelevant antibody (Fig. 1*b, lane 5*). It should be noted that the efficacy of immunoprecipitation of $mGlu_{1\alpha}$ receptor by the anti-adenosine receptor antibody was much less than when an anti-$mGlu_1$ receptor antibody was used. Overall, these results indicate that there are zones in which $mGlu_{1\alpha}$ and A1 receptors do not co-distribute and that there are zones in which the two receptors co-distribute and form aggregates (heteromers).

Interaction of $mGlu_{1\alpha}$ and A1 Receptors in Transiently Transfected HEK-293 Cells—The close association of $mGlu_{1\alpha}$ and A1 receptors was subsequently studied in co-transfected HEK-293 cells by co-immunoprecipitation and double immunolabeling experiments. By confocal microscopy analysis of HEK-293 cells transiently transfected with the cDNAs for $mGlu_{1\alpha}$-FLAG and A1 receptors, a marked overlap in the distribution of the two proteins was found (Fig. 2). As deduced from horizontal optical sections of permeabilized (+*Triton*) and nonpermeabilized (−*Triton*) cells, co-localization was not restricted to the plasma membrane but extended to intracellular compartments. Interestingly, when the double immunolabeling experiment was performed on HEK-293 cells transiently transfected with the cDNAs for $mGlu_{1\beta}$-FLAG, the C-terminal splice variant of $mGlu_1$ receptor (Fig. 4*a*), and A1 receptors, no co-localization between both proteins was observed at the plasma membrane level (Fig. 2), suggesting a specificity for the co-localization between $mGlu_{1\alpha}$-FLAG and A1 receptors.

From co-transfected HEK cell extracts, the antibody against A1R (PC11-Ab) co-immunoprecipitated a band of 150 kDa, which corresponds to the $mGlu_{1\alpha}$-FLAG receptor (Fig. 3, *lane 5*). This band did not appear in immunoprecipitates from cells transfected with the cDNA for either A1 receptors (Fig. 3, *lane 4*) or $mGlu_{1\alpha}$ (Fig. 3, *lane 6*) or when an irrelevant antibody was used (data not shown). Conversely, when we immunoprecipitate with the FLAG antibody to pull down $mGlu_{1\alpha}$-FLAG receptor in the transiently co-transfected HEK-293 cells, and subsequently the immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using anti-A1R antibodies, a band which corresponds to the A1R was observed (Fig. 3, *lane 11*). Interestingly, no immunoprecipitation of $mGlu_{1\beta}$ receptor, the C-terminal splice variant of $mGlu_1$ receptor (Fig. 4*a*), was obtained from co-transfected cells using the antibody against A1R (PC11-Ab) (Fig. 4*b, lane 7*). Additionally, the construct $mGlu_1$-M7, a deleted mutant close to the splice variant site of the $mGlu_1$ receptor (Fig. 4*a*), also did not interact with A1R (Fig. 4*b, lane 8*). Overall, these results suggest that the C-terminal tail of the $mGlu_{1\alpha}$ receptor (Fig. 4*a*) is implicated in the interaction of both receptors.

To test the functional significance of an $mGlu_{1\alpha}$/A1 receptors interaction, measurements of calcium mobilization in co-transfected HEK-293 cells were performed. In HEK cells transiently expressing A1R or A1R plus $mGlu_{1\alpha}$-FLAG R-PIA mobilized intracellular calcium in a concentration-dependent manner, with an EC_{50} value of 46.8 ± 4.4 nM for the single expressing A1R cells or 45.7 ± 5.1 nM for the doubly A1R plus $mGlu_{1\alpha}$-FLAG expressing cells. On the other hand, in these cells quisqualic acid also mobilized intracellular calcium in a concentration-dependent manner, with an EC_{50} value of 4.1 ± 1.1 μM for the single expressing $mGlu_{1\alpha}$-FLAG cells or 5.6 ± 1.1 μM for the doubly A1R plus $mGlu_{1\alpha}$-FLAG expressing cells. The densities of the transiently expressed receptors were controlled by

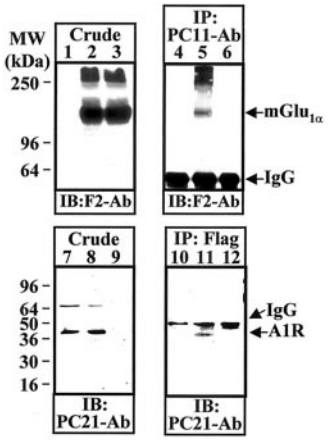

FIG. 3. **Interaction of $mGlu_{1\alpha}$ and A1 receptors in transiently transfected HEK-293 cells.** Cells transiently expressing A1R alone (*lanes 1* and *7*), A1R plus $mGlu_{1\alpha}$–FLAG (*lanes 2* and 8), or $mGlu_{1\alpha}$-FLAG alone (*lanes 3* and *9*) were washed and solubilized in ice-cold lysis buffer and processed for immunoprecipitation using anti-A1R antibodies (PC11-Ab; 10 μg/ml; *lanes 4–6*) and anti-FLAG monoclonal antibody (Sigma, Clone M2; 10 μg/ml; *lanes 10–12*). Solubilized membranes (*Crude*, *lanes 1–3* and *7–9*) and immunoprecipitates (*IP*, *lanes 4–6* and *10–12*) were analyzed by SDS-PAGE and immunoblotted (*IB*) using anti-$mGlu_{1\alpha}$ receptor antibodies (F2-Ab; 5 μg/ml) or anti-A1R antibodies (PC21-Ab; 10 μg/ml). Immunoreactive bands were detected as before. IgG indicates the position of the immunoglobulins used in the immunoprecipitation.

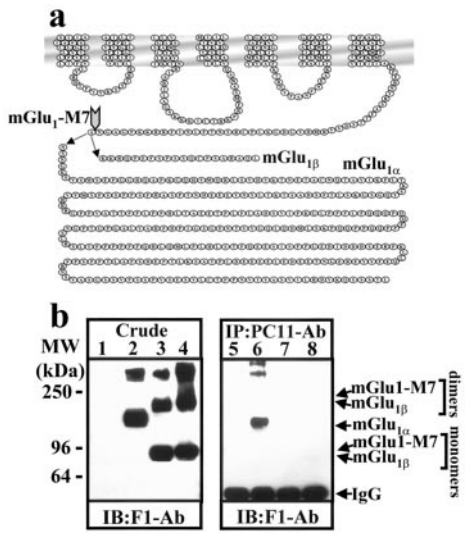

FIG. 4. **Specificity of the interaction between $mGlu_{1\alpha}$ and A1 receptors in transiently transfected HEK-293 cells.** *a,* schematic representation of the primary structure of rat $mGlu_{1\alpha}$ and $mGlu_{1\beta}$ receptors. The *gray arrowhead* indicates the position of the stop codon introduced to generate the deleted mutant $mGlu_1$-M7. *b,* HEK-293 cells transiently expressing A1R alone (*lane 1*), A1R plus $mGlu_{1\alpha}$–FLAG (*lane 2*), A1R plus $mGlu_{1\beta}$-FLAG (*lane 3*), and A1R plus $mGlu_1$-M7-FLAG (*lane 4*) were processed for immunoprecipitation using anti-A1R antibodies (PC11-Ab; 10 μg/ml). Solubilized membranes (*Crude*, *lanes 1–4*) and immunoprecipitates (*IP*, *lanes 5–8*) were analyzed by SDS-PAGE and immunoblotted (*IB*) using anti-$mGlu_1$ antibodies (F1-Ab; 5 μg/ml). Immunoreactive bands were detected as before. IgG indicates the position of the immunoglobulins used in the immunoprecipitation.

means of ligand binding experiments performed in these cells (see "Experimental Procedures"). Cells transfected with the cDNA for A1R alone express 3.4 ± 0.4 pmol of A1R/mg protein as detected by [^{3}H]R-PIA binding with a K_D of 27 ± 3 nM. In these cells the A1R agonist R-PIA leads to a calcium peak (Fig. 5). On the other hand, cells transfected only with the cDNA for $mGlu_{1\alpha}$ receptor express 3.3 ± 0.9 pmol of $mGlu_{1\alpha}$ receptor/mg

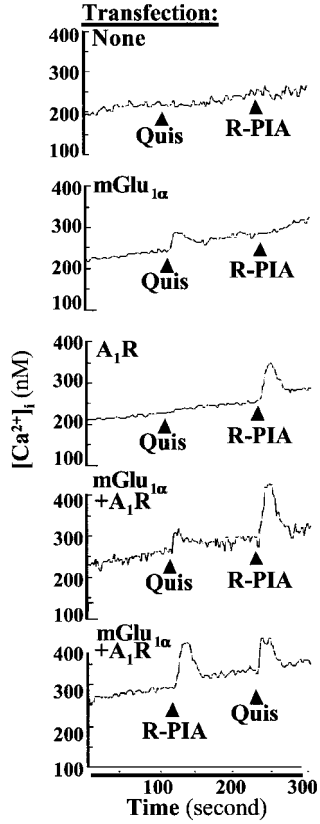

FIG. 5. **Ca^{2+} mobilization in HEK-293 transiently expressing $mGlu_{1\alpha}$ and A1 receptors.** Intracellular Ca^{2+} concentrations were measured in suspended cells loaded with FURA-2/AM after stimulation with the $mGlu_{1\alpha}$ receptor agonist quisqualic acid (100 μM) (*Quis*) or A1R agonist N^6-(*R*)-phenylisopropyladenosine (500 nM) (*R-PIA*).

protein as detected by [^{3}H]quisqualic binding with a K_D of 177 ± 46 nM, and a weak calcium peak was detected when treated with the agonist for the metabotropic glutamate receptor, quisqualic acid (Fig. 5). Interestingly, in HEK-293 cells co-transfected with both receptors (3.3 ± 0.2 pmol of A1R/mg of protein and 2.8 ± 1.5 pmol of $mGlu_{1\alpha}$/mg of protein), preincubation with quisqualic acid markedly potentiated the calcium peak obtained in response to A1R activation (140 ± 10%; n = 3). Conversely, preincubation of co-transfected cells with the agonist for A1R led to a marked enhancement of the signal provided by quisqualic acid (180 ± 20%; n = 3) (Fig. 5). Quisqualic acid or R-PIA failed to bind or to provide any signal in nontransfected HEK-293 cells. These results clearly show a heterologous sensitization or synergistic effect upon $mGlu_{1\alpha}$ and A1 receptors activation.

Interaction of $mGlu_{1\alpha}$ and A1 Receptors in Primary Rat Cortical Neurons—To assess the physiological relevance of the $mGlu_{1\alpha}$/A1 receptors interaction, we analyzed the distribution of both receptors in primary rat cortical neurons. Both receptors showed a similar punctate distribution throughout the proximal and distal dendrites, and the degree of co-localization at these locations was very high (Fig. 6, *a–c*). In fact, some of the $mGlu_{1\alpha}$ receptor– or A1R-containing puncta co-distributed with the synaptic marker protein synaptophysin (Fig. 6, *d–i*), suggesting that they could be localized to synapses. Thus, synapses are one of the specific cellular sites where $mGlu_{1\alpha}$ and A1 receptors interact.

Glutamate/adenosine receptors interaction may be important for modulating the role of $mGlu_{1\alpha}$ receptor in neurodegeneration/neuroprotection, an issue that is still controversial. When examining this role a number of factors, including the

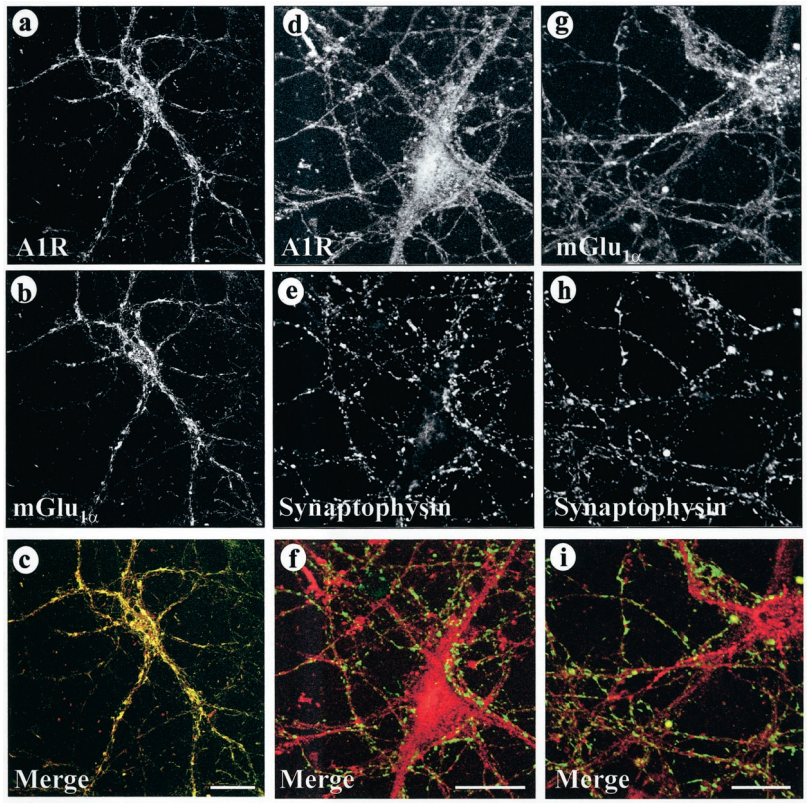

FIG. 6. **Co-localization of mGluR1α and A1R in primary cultures of rat cortical neurons.** Neurons (days in vitro 14–21) were fixed, permeabilized, and processed for immunostaining using rhodamine-conjugated anti-A1R antibody (PC21-Ab; 15 μg/ml) and fluorescein-conjugated anti-mGlu$_{1\alpha}$ receptor antibody (F2-Ab; 15 μg/ml) (*a–c*), anti-A1R antibody (PC21-Ab, 5 μg/ml) and anti-synaptophysin monoclonal antibody (1/20) (*d–f*), or anti-mGlu$_{1\alpha}$ receptor antibody (F2-Ab; 5 μg/ml) and anti-synaptophysin monoclonal antibody (1/20) (*g–i*). Primary antibodies in *d–i* were detected using fluorescein-conjugated donkey anti-mouse IgG antibody (1/50) or Texas Red-conjugated donkey anti-rabbit (1/50)(*d–i*). Cells were analyzed by double immunofluorescence with a confocal microscopy. Images show A1R receptor (*a*) in *red* mGlu$_{1\alpha}$ receptor (*b*) in *green*, A1R (*d*) and mGlu$_{1\alpha}$ (*g*) receptors in *red*, and synaptophysin (*h*) in *green*. Superimposition of images reveals co-localization in *yellow* (*c, f*, and *i*). *Scale bar*, 10 μm.

heteromeric composition of NMDA receptors, the time of exposure to drugs or to ambient glutamate, and the function of astrocytes clearing extracellular glutamate and producing neurotoxic or neuroprotective factors must be taken into account (20). On the other hand, glutamate could also modulate the well known function of adenosine as neuroprotective factor (12). It is thus likely that the interaction of mGlu$_{1\alpha}$/A1 receptors could be beneficial in situations of enhanced neuronal activity, in which potentiation of postsynaptic adenosine A1 receptor limits evoked depolarization and results in decreased activation of voltage-dependent Ca^{2+} channels and NMDA receptor ion channels, through which Ca^{2+} enters cell bodies (30). We have therefore examined the effect of activating of both mGlu$_1$ and A1 adenosine receptors on NMDA-mediated neurotoxicity in primary neuronal cultures. In agreement with previously described data (20), submaximal concentrations of NMDA induced neuronal death, which was enhanced by the presence of quisqualic acid during the NMDA treatment (Fig. 7). In contrast, when the adenosine A1 receptor agonist, R-PIA, was present during the NMDA treatment, the induced neurotoxicity was reduced by nearly 50%. When added simultaneously, R-PIA reduced the enhancement of the neurotoxicity induced by quisqualic acid (Fig. 7). On the other hand, pre-exposure of neurones to R-PIA or to quisqualic acid also reduced the NMDA-induced neurotoxicity. This reduction was more marked if both quisqualic acid and R-PIA were present during the pre-exposure, showing that the simultaneous activation of both receptors appears to increase the protection of the neurones against the NMDA treatment compared with the effect evoked by either quisqualate or R-PIA applied separately. These results show the relevance of the interaction of mGlu$_{1\alpha}$/A1 receptors and support the concept of specificity and complexity of this interaction, being the spatiotemporal segregation profile of adenosine/glutamate during synaptic activity of special importance to achieve a neuroprotective or a neurotoxic effect.

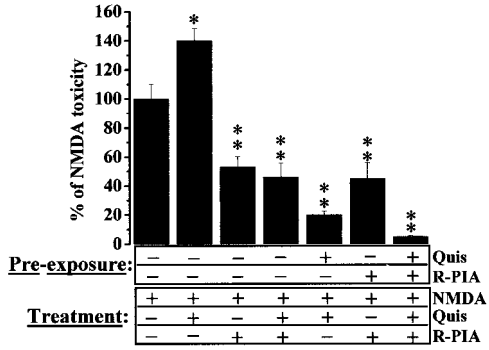

FIG. 7. **Excitotoxicity in rat cortical neurons.** Cortical neurons were exposed for 10 min to 30 μM of NMDA. Quisqualic acid (*Quis,* 100 μM) and/or R-PIA (100 nM) were transiently applied (for 1 min) 5 min prior to the addition of NMDA (*Pre-exposure*) and/or added together with NMDA for 10 min (*Treatment*). Quisqualic acid treatments were always made in the presence of 50 μM 6-cyano-7-nitroquinoxaline-2,3-dione (*CNQX*, Sigma) to block non-NMDA receptors. Death neurons were computed by assessing the propidium iodide staining in photomicrographs of 10 representative fields from each monolayer of treated neurons and expressed as percent of NMDA toxicity. *Asterisks* denote differences from the control (*, $p < 0.05$; **, $p < 0.01$, two-tail *t* test).

DISCUSSION

Here we describe a novel interaction between two unrelated G protein-coupled receptors (GPCR), namely the metabotropic glutamate type 1α and the adenosine A1 receptors. Although the cooperativity of the agonist binding to GPCR suggested the possibility of oligomerization of G proteins and their associated receptors (31), the existence and the precise function of GPCR homo- or heteromerization has not been fully elucidated.

Metabotropic glutamate type 1α and adenosine A1 receptors show a high degree of co-localization in co-transfected cells and in more physiological systems as neurons from rat cerebellum and from rat cortex. Both receptors co-immunoprecipitate from co-transfected cells and from rat cerebellum synaptosomes,

suggesting that $mGlu_{1\alpha}$ and A1 receptors interact and that this interaction is physiologically relevant. From a functional point of view, heteromerization often results in facilitatory responses or synergistic effects. Thus, GABABR1 and GABABR2 receptors need to be co-expressed and assembled into heteromers to reach the cell surface (32–35). On the other hand, two functional opioid receptors, κ and δ, can heteromerize, which changes the pharmacology of the individual receptors and potentiates signal transduction (36). Also, it has been described for heterodimerizations for the CC chemokine receptor 2 (CCR2) and the CX chemokine receptor 4 (CXCR4) and/or CC chemokine receptor 5 (CCR5) (37). Recently, it has been demonstrated that κ and δ opioid receptors can form heteromers with β_2-adrenergic receptors affecting their trafficking properties without any significant alteration in the ligand binding or coupling properties of the receptors (38). There are also some papers reporting similar interactions between GPCR and non-GPCR proteins, for example the receptor activity-modifying protein, a small protein containing only one putative transmembrane domain and a short cytosolic tail, acting as a chaperone protein that facilitates the cell surface targeting and modulator function of the calcitonin-receptor-like receptor (39). Interestingly, recent data (40) indicated that a GPCR, the D5-dopamine receptor, physically interacts with the γ-aminobutyric acid A (GABAa) receptor, a GABA-operated Cl^- channel. This physical interaction was dependent on the presence of agonist for both receptors and necessary for the functional cross-talk between the D5 and GABAa receptors (40). In the case of cells co-expressing $mGlu_{1\alpha}$ and A1 receptors, a glutamate/adenosine synergism was found at the level of calcium mobilization (Fig. 5). Furthermore, in experiments of neuroprotection performed in neuronal cultures, preincubation with quisqualic acid plus adenosine was much more effective than pretreatment with any of the compounds. These results suggest that activation of $mGlu_{1\alpha}$ and A1 receptors in the same neuron results in synergism. Heteromerization, however, does not always lead to facilitation or synergistic events. Thus, in basal ganglia, there is an adenosine-mediated antagonism of dopaminergic neurotransmission. This antagonism is in part due to cross-talk at the level of second messengers but is also mediated by formation of adenosine/dopamine receptor heteromers (41). Although it is difficult to ascertain to what extent the antagonism is mediated by heteromerization or to interference in signaling, there is evidence indicating that both events operate and are closely interrelated. Thus, in cells where A1R and D1-dopamine receptors are present, as in nigrostriatal GABAergic neurons, adenosine leads to both the disappearance of the high affinity site of D1 receptors, probably via conformational changes in A1R/D1R heteromers, and a reduction in dopamine-induced cAMP increases, an effect due to cross-talk at the adenylate cyclase level (41).

There is experimental and molecular modeling evidence that intramembrane domains are involved in the formation of homodimers of G protein-coupled receptors. Gouldson *et al.* (42) have hypothesized that domain swapping with involvement of transmembrane regions 5 and 6 is responsible for homo- and heteromerization of G protein-coupled receptors. In contrast, heterodimerization of GABAR1 and GABABR2 receptors is mediated by the coiled-coil interaction of the C-terminal cytoplasmic tails (35). In the case of $mGlu_{1a}$/A1 receptors' heteromers the interaction depends on the C terminus of $mGlu_{1\alpha}$ receptor as its splice variant, $mGlu_{1\beta}$ receptor, which has a short and different C terminus, does not interact with A1R. Also a deleted mutant of $mGlu_1$ receptor, close to the splice variant site and missing nearly all the C terminus of the receptor, does not interact with A1R. The co-immunoprecipitation of $mGlu_{1\alpha}$ and A1 receptors might be due to a physical association between them, but it also could be the case that both receptors are recruited into a specific signaling complex at specific synapses via common interactions with other proteins.

One possible mechanism for this $mGlu_{1\alpha}$/A1 receptor coupling is that it is directed by interactions of the cytoplasmic C terminus with specific targeting proteins. This type of targeting mechanism appears to operate for the synaptic localization of the ionotropic glutamate receptors and a number of different proteins, containing PDZ domains, which interact with specific C-terminal sequences of these receptors (43–47). Also, the EVH1-like domain (ENA/VASP homology domain 1)-containing protein, which binds specifically to the C-terminal residues of mGlu1α receptor, has been described (48). This protein, termed Homer-1A, was isolated as a synaptic plasticity-regulated gene from rat hippocampus (48, 49). Additional proteins related to Homer-1A have also been described, namely Homer-1B, Homer-1C, Homer-2A, Homer 2B, Homer-2C, Homer-2D, Homer-3A, Homer-3B, Homer-3C, and Homer-3D (50–53). The ability of Homer to link $mGlu_{1\alpha}$ receptor to Shank, a scaffolding multimeric signaling protein, may contribute to anchoring the $mGlu_{1\alpha}$ receptor to specific sites at the plasma membrane (8, 9). Our efforts are directed to find a network of protein interactions that are shared by both receptors and that likely play a key role in the signaling mechanisms of both $mGlu_{1\alpha}$ and A1 receptors. The nature of the signaling complexes formed would depend on the type of receptors present in a given neuron, and the effect will be determined by the balance between concentrations of agonists in the synaptic cleft and timing of receptor activation. It has been proposed that the mechanism of the biphasic effects of quisqualic acid to increase NMDA toxicity if added together with NMDA or to reduce it if pretreated before NMDA is in part due to the functional switch described for the $mGlu_{1\alpha}$ receptor (54, 55, 20). In mixed cortical or pure hippocampal neuronal cultures a first application of 3,5-dihydroxyphenylglycine, an agonist of the group I mGlu receptors, potentiated toxicity induced by submaximal concentrations of NMDA, whereas the same drug applied shortly after a brief pre-exposure, protected against neuronal death (20). Interestingly, the switch in the regulation of excitotoxic neuronal death was sensitive to protein kinase C inhibitors (20). This mechanism may explain the opposite results obtained with group I mGlu receptors, assuming that the influence of group I mGlu receptors on excitotoxic neuronal death will depend on the "functional status" of group I mGlu receptors (naive *versus* experienced or unphosphorylated *versus* phosphorylated receptors) (20). In Fig. 7 we show that the timing in $mGlu_{1\alpha}$ and A1 receptors activation is very important to achieve a maximum effect in adenosine- and glutamate-mediated neuroprotection/neurodegeneration. In fact, although adenosine added together with glutamate was protective for cultured neurons, this protection was nearly total by preincubation with the metabotropic glutamate receptor agonist.

Our data provide biochemical and functional evidence for $mGlu_{1\alpha}$ and A1 receptor-receptor interaction. The way A1R is involved in interactions with receptors for other neurotransmitters, using different receptor system to synchronize synaptic transmission, opens new perspectives to understand the actual role of this autacoid. Since $mGlu_{1\alpha}$ receptors seem to be involved in the pathophysiology of neuropsychiatric diseases such as Alzheimer's and related disorders, this molecular interaction offers a new basis for the design of novel strategies to study the genesis and evolution of these diseases and of novel agents to treat them.

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