

Hetero-oligomerization of adenosine A₁ receptors with P2Y₁ receptors in rat brains

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Abstract Adenosine and ATP modulate cellular and tissue functions via specific P1 and P2 receptors, respectively. Although, in general, adenosine inhibits excitability and ATP functions as an excitatory transmitter in the central nervous system, little is known about the direct interaction between P1 and P2 receptors. We recently demonstrated that the G_{i/o}-coupled adenosine A₁ receptor (A₁R) and G_{q/11}-coupled P2Y₁ receptor (P2Y₁R) form a heteromeric complex with a unique pharmacology in cotransfected HEK293T cells using the coimmunoprecipitation of differentially epitope-tagged forms of the receptor [Yoshioka et al. (2001) *Proc. Natl. Acad. Sci. USA* 98, 7617–7622], although it remained to be determined whether this hetero-oligomerization occurs in vivo. In the present study, we first demonstrated a high degree of colocalization of A₁R and P2Y₁R by double immunofluorescence experiments with confocal laser microscopy in rat cortex, hippocampus and cerebellum in addition to primary cultures of cortical neurons. Then, a direct association of A₁R with P2Y₁R was shown in coimmunoprecipitation studies using membrane extracts from these regions of rat brain. Together, these results suggest the widespread colocalization of A₁R and P2Y₁R in rat brain, and both receptors can exist in the same neuron, and therefore associate as hetero-oligomeric complexes in the rat brain.

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1. Introduction

Adenosine and ATP mediate a wide variety of physiological processes, including smooth muscle contraction, immune response, platelet aggregation, pain, cardiac function, neuromodulation and neurotransmission via purinergic receptors, which are divided into P1 and P2 receptors [1]. Molecular cloning and pharmacological studies have identified four types

of P1 receptors, i.e. adenosine A₁, A_{2A}, A_{2B} and A₃ receptors, which are G protein-coupled receptors (GPCRs). P2 receptors are subclassified into P2X and P2Y types. Seven mammalian P2X receptors that are ligand-gated ion channels and six mammalian P2Y receptors (P2Y₁, 2, 4, 6, 11, 12), all of which are GPCRs, have been cloned.

The adenosine A₁ receptor (A₁R) is functionally coupled to members of the pertussis toxin (PTX)-sensitive family of G proteins, G_{i/o} proteins, and its activation regulates several effectors such as adenylyl cyclase [1]. On the other hand, it was established that the P2Y₁ receptor (P2Y₁R) stimulates phospholipase C through PTX-insensitive G protein, G_{q/11} [1]. Recent work by Mendoza-Fernandez et al. [2] has demonstrated that ATP inhibited the synaptic release of glutamate in rat hippocampal neurons by direct activation of P2Y receptors that are PTX- and theophylline derivative (P1 receptor antagonist)-sensitive, and P2 receptor antagonist-insensitive. These results suggest that there is a P1-like P2Y receptor coupled to the G_{i/o} proteins in rat hippocampal neurons, although its identity is unclear.

We have recently reported that cotransfection of A₁R and P2Y₁R in HEK293T cells results in the generation of a unique adenosine receptor with P2Y-like agonistic pharmacology via heteromeric complexes between A₁R and P2Y₁R [3]. Such a hetero-oligomer represents a novel structure for a purinergic receptor, which may help to explain the undefined physiological functions of ATP and adenosine in the nervous system [4–7]. We also detected the constitutive hetero-oligomerization of A₁R/P2Y₁R in transfected living cells by bioluminescence resonance energy transfer [8]. However, clear evidence for the oligomerization of these two purinergic receptors in vivo has not been provided yet.

The aim of the present study was to demonstrate A₁R/P2Y₁R oligomerization in vivo in various regions of the rat brain. Double immunostaining coupled with confocal laser microscopic imaging was used to demonstrate the colocalization of A₁R and P2Y₁R in the same cells. Coimmunoprecipitation experiments were also used to confirm the direct association of A₁R and P2Y₁R in the extract of brain tissues.

2. Materials and methods

2.1. Primary cultures

Cerebral cortices were dissected out from rat embryos (18 embryonic days) and cultured as described previously [9]. Briefly, after the removal of meninges, tissues were dissociated by mechanical trituration following digestion with 0.15 U/ml papain (in phosphate-buffered saline (PBS) containing 0.02% L-cysteine, 0.02% bovine serum albu-

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Abbreviations: A₁R, adenosine A₁ receptor; P2Y₁R, P2Y₁ receptor; GPCR, G protein-coupled receptor

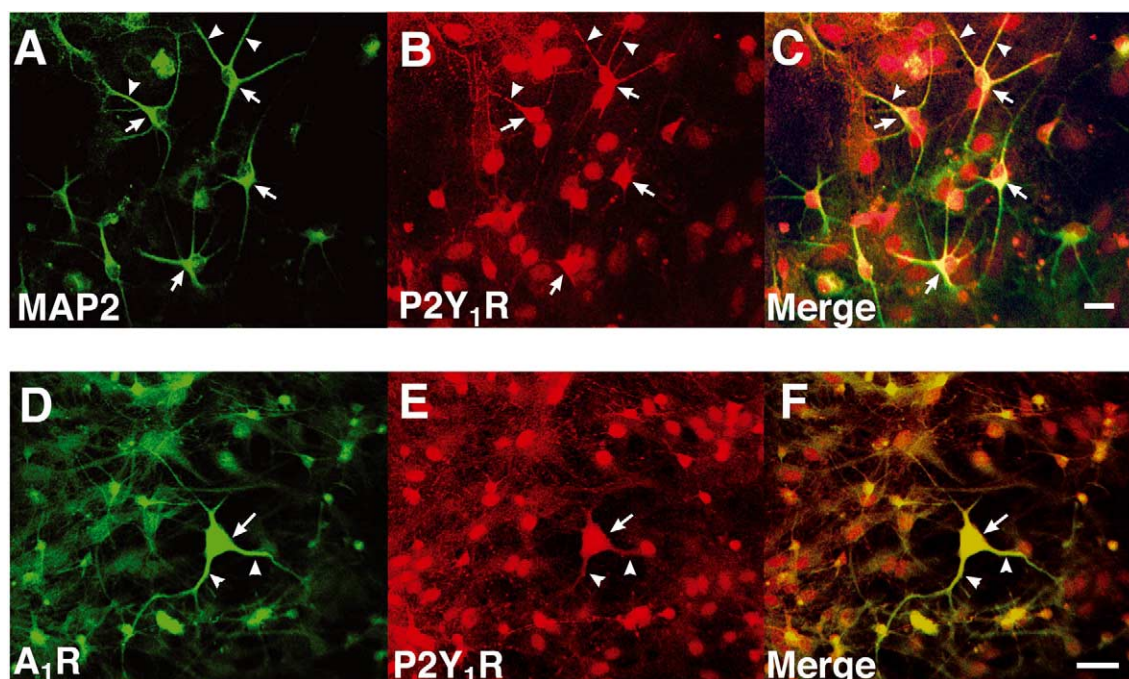


Fig. 1. Double immunofluorescence staining and confocal images of rat cortical cultured neurons. A–C: Staining of MAP2 (A, green image) and P2Y₁R (B, red image) in rat cortical cultured cells, indicating P2Y₁Rs express neural soma and dendrites. Superimposition of images reveals MAP2 and P2Y₁R colocalization in yellow (C). D,E: Staining of A₁R (D, green image) and P2Y₁R (E, red image) in rat cortical cultured cells. Superimposition of images reveals A₁R and P2Y₁R colocalization in yellow (F). The images show a single horizontal optical section of representative cells. Scale bar, 50 μ m. Arrows and arrowheads indicate the representative position of colocalization for these receptors.

min, and 0.5% glucose). Dissociated neurons were plated on polyethyleneimine-coated coverslips and cultured with Dulbecco's modified Eagle's medium supplemented with 5% horse serum, 5% newborn calf serum, and 1 mM sodium pyruvate. The culture was maintained in a humidified atmosphere of 93% air and 7% CO₂ at 37°C and used for immunostaining after 21–28 days.

2.2. Double immunostaining

Primary cultures: for immunocytochemistry, primary cultures of rat cortical neurons were fixed in 4% paraformaldehyde for 15 min and washed with PBS containing 20 mM glycine to quench the aldehyde groups. Cells were permeabilized with 0.2% Triton X-100 for 5 min in PBS, rinsed with PBS, and treated with 5% normal goat serum in PBS for 30 min at room temperature. Double immunostaining was performed with anti-MAP2 monoclonal antibody (1:500, Roche), anti-A₁R mouse monoclonal antibody (1 μ g/ml) and anti-P2Y₁R rabbit polyclonal antibody (1 μ g/ml) for 90 min at 37°C. The characterization of these antibodies was previously reported [10,11]. Cells were washed and stained with Alexa 488-conjugated goat anti-mouse IgG antibody (1:200, Molecular Probe) for MAP2 and A₁R, or Alexa 568-conjugated goat anti-rabbit IgG antibody for P2Y₁R (1:200, Molecular Probe). The specificity of the conjugates was confirmed by abolition of detectable staining when the primary antibody was omitted. Cells on chamber slides were rinsed with PBS and mounted with FluoroGuard (Bio-Rad). Confocal laser microscopy was performed with a Carl Zeiss LSM410 using a 5- μ m optical slice.

Brain sections: for immunohistochemistry, adult male rats (Wistar) were deeply anesthetized with Nembutal, and blood was removed from tissues by intracardiac perfusion with 0.9% NaCl for 2 min. Tissues were then fixed *in situ* by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB) for 20 min and the brains were dissected out. After 16 h post-fixation, brain tissues were replaced with 30% sucrose in PB at 4°C overnight. Serial 40- μ m-thick cryostat sagittal sections were cut (Leica). Floating sections were incubated with 0.5% Triton X-100 in PB (T-PB) for 30 min and treated with 5% normal goat serum in PB (blocking solution), and then with primary antibodies for 72 h at 4°C. Primary antibodies [10–11] were mouse monoclonal anti-A₁R antibody (1 μ g/ml, diluted in blocking solution) and rabbit polyclonal anti-P2Y₁R antibody (1 μ g/

ml, diluted in blocking solution). Sections were then washed with T-PB before incubation with secondary conjugates. Conjugates used were Alexa 488-conjugated goat anti-mouse IgG antibody (1:200, Molecular Probe), and Alexa 568-conjugated goat anti-rabbit IgG antibody (1:200, Molecular Probe). Sections were washed with T-PB, coverslipped with FluoroGuard (Bio-Rad), and sealed with nail polish. Fluorescent images were collected using a Zeiss LSM410 confocal laser microscope with a 10- μ m optical slice.

2.3. Immunoprecipitation and Western blotting

Cortical, hippocampal and cerebellar tissues were dissected out from adult male rat brains (Wistar). The tissues were then disrupted with a Polytron homogenizer for 5-s periods in 50 mM Tris-acetate, pH 7.4, containing a protease inhibitor cocktail (Roche Diagnostics), and the resulting cell suspensions were centrifuged at 30 000 $\times$ g for 30 min at 4°C. The precipitated cell membranes were washed twice with the homogenizing buffer. They were then solubilized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 300 mM NaCl and a protease inhibitor cocktail) for 60 min at 4°C on a rotator. The mixture was centrifuged at 18 500 $\times$ g for 20 min at 4°C, and the supernatant was precleared with Protein G-agarose (Roche Diagnostics). Subsequently, the precleared lysate was incubated with rabbit polyclonal anti-A₁R antibody (1 μ g/ml, Sigma) for 60 min at 4°C on a rotator, and then Protein G-agarose was added to the mixture. The incubation was continued for an additional 120 min. The Protein G-agarose was recovered by centrifugation and washed three times with the same lysis buffer. Immunoprecipitates were eluted from the Protein G-agarose with SDS-PAGE sample buffer, resolved by 12% SDS-PAGE, and electrotransferred to a nitrocellulose membrane. Receptors on the blot were detected using anti-A₁R or anti-P2Y₁R antibody, followed by horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG antibody, respectively. The reactive bands were visualized with enhanced chemiluminescent substrates (SuperSignal, Pierce) [3]. It was confirmed that immunoprecipitation with plain rabbit IgG revealed no detectable receptor bands by Western blotting.

Transfection of HEK293T cells with A₁R or P2Y₁R cDNA and the immunoprecipitation experiments of the transfected HEK293T cells were performed as described previously [3].

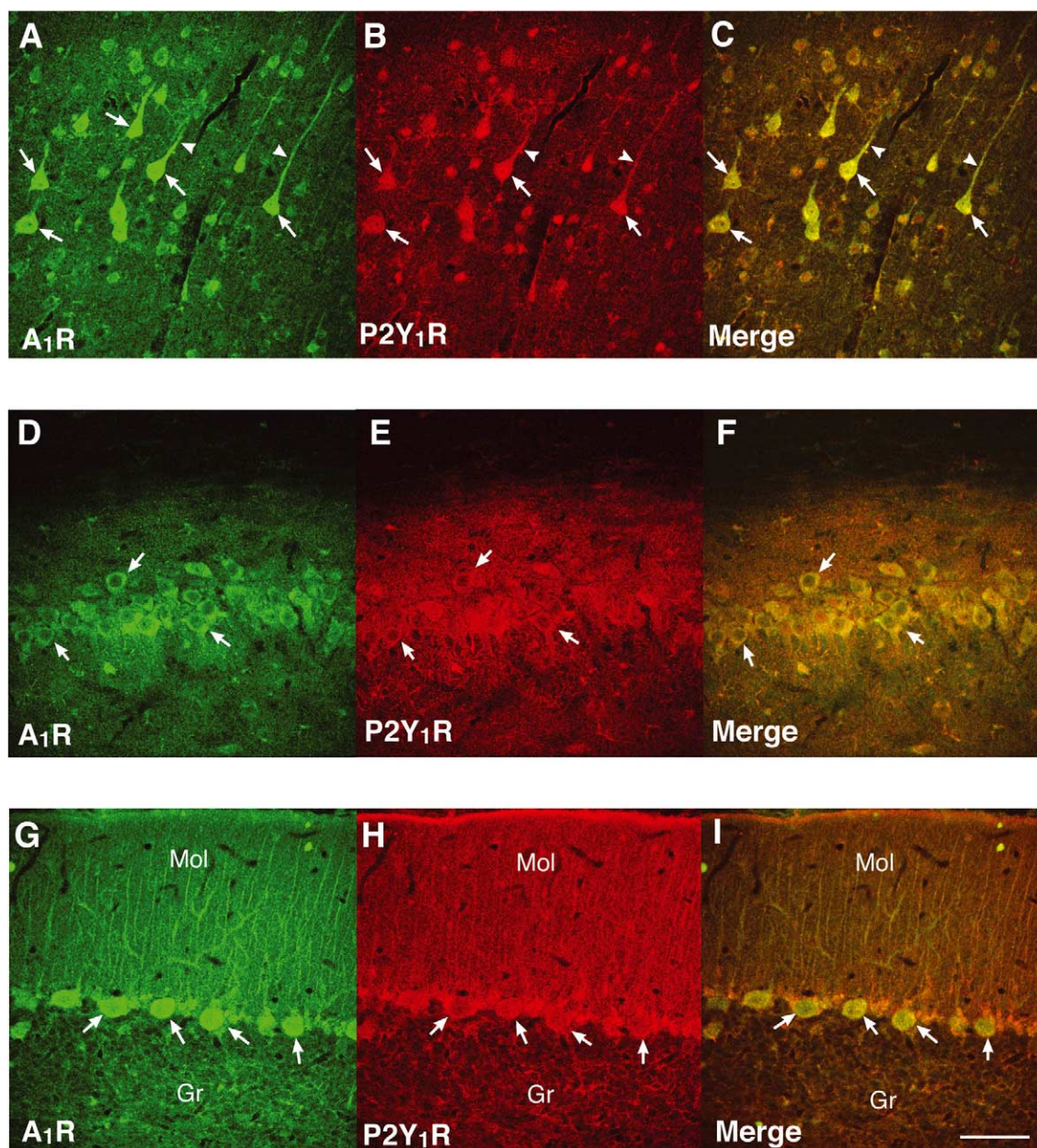


Fig. 2. Double immunofluorescence staining and confocal images of rat brain regions. A_1R and $P2Y_1R$ immunoreactivity was detected in the brain section of cortex (A–C), hippocampus (D–F) and cerebellum (G–I) from rats ($n=3-5$). Confocal microscopy revealed colocalization (yellow in C, F, I) of A_1R (green in A, D, G) and $P2Y_1R$ (red in B, E, H) in cortical pyramidal neurons of layer V (A–C, arrows) and dendrites (A, arrowheads), in hippocampal pyramidal cell layer (D–F, arrows), in cerebellar Purkinje cells (G–I, arrows) and the molecular layer (G–I, Mol). Scale bar, 100 μm . Arrows indicate the representative position of colocalization for these receptors.

3. Results

3.1. Double immunolabeling of A_1R and $P2Y_1R$ in the rat brain

Colocalization of A_1R and $P2Y_1R$ was studied in primary cultures of rat cortex. Images of rat cortical primary neurons labeled for MAP2 (green) or A_1R (green) and $P2Y_1R$ (red) are shown in Fig. 1. The specificity of the antibodies against A_1R and $P2Y_1R$ was confirmed by the immunocytochemistry of recombinant receptor-expressing cell lines, i.e. antibodies used in this study showed no cross-labeling in A_1R - and $P2Y_1R$ -transfected HEK293T cells (data not shown). Omission of the primary antibody achieved no significant staining (data not shown). As shown in Fig. 1 (panels A–C), there is a

clear codistribution of $P2Y_1R$ and MAP2 immunoreactivity in several neurons, which is consistent with the previous reports [11,12]. A_1R and $P2Y_1R$ immunoreactivity was predominantly detected in soma and dendrites in the cortical cultured neurons (Fig. 1D,E). Double staining analysis revealed substantial colocalization of both receptors at the soma and dendrites of several cortical neurons (yellow in Fig. 1F).

Immunohistochemical studies showed that immunoreactivity for both A_1R and $P2Y_1R$ is present in cortex, hippocampus and cerebellum (Fig. 2). Their expression is mainly restricted to the cell bodies and the dendrites of neural cells. This observation is in agreement with previous reports on the distribution of these receptors in rat or human brain [10–12]

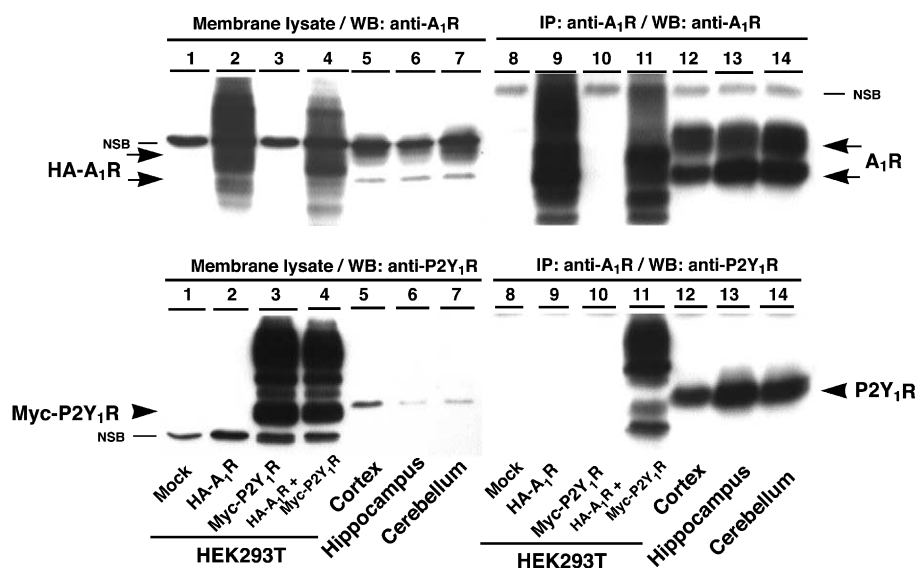


Fig. 3. Coimmunoprecipitation of adenosine A_1 Rs and $P2Y_1$ Rs from rat cortex, hippocampus and cerebellum. Extracts from various regions were immunoprecipitated with anti- A_1 R antibodies, and analyzed by Western blotting with anti- A_1 R (upper panels) or anti- $P2Y_1$ R (lower panels) antibodies. As a positive control, Myc- $P2Y_1$ R was coimmunoprecipitated by anti- A_1 R antibodies along with HA- A_1 R from the HEK293T cells coexpressing HA- A_1 R/Myc- $P2Y_1$ R (lane 11). Anti- A_1 R antibodies precipitated A_1 R (upper panel, M_r = 33, 39 kDa) along with $P2Y_1$ R (lower panel, M_r = 62 kDa) from membrane lysates of rat cortex (lane 12), hippocampus (lane 13) and cerebellum (lane 14). NSB, non-specific band; arrows, A_1 R or HA- A_1 R; arrowheads, $P2Y_1$ R or Myc- $P2Y_1$ R. Data are representative of three independent experiments.

and also with our present observation in rat cortical cultured neurons (Fig. 1). A_1 R and $P2Y_1$ R immunoreactivity was present throughout the cortical layers, with a strongly defined border between layers IV and V (Fig. 2A,B). Merged immunoreactivity for A_1 R and $P2Y_1$ R showed a codistribution of these receptors in large pyramidal cells located in layers V (arrows, Fig. 2C). In the hippocampal formation, A_1 R and $P2Y_1$ R immunoreactivity were widely distributed (Fig. 2D,E). Pyramidal cell bodies were significantly stained with both antibodies for A_1 R and $P2Y_1$ R (yellow in Fig. 2F). Purkinje cell layers in the cerebellum were clearly labeled by antibodies for both receptors (Fig. 2G,H). Weaker immunostaining for both A_1 R and $P2Y_1$ R was detected in the granule cell layers (Gr) and in the molecular cell layers (Mol) (Fig. 2G–I). When the images were merged, a yellow color results from colocalization of A_1 R and $P2Y_1$ R over the Purkinje cells and molecular cell layers (yellow in Fig. 2I).

3.2. Coimmunoprecipitation of A_1 R and $P2Y_1$ R from rat brain

The codistribution of A_1 R and $P2Y_1$ R immunoreactivity in several regions of the rat brain described above suggests a potential interaction between these two receptors in precise areas. The existence of A_1 R/ $P2Y_1$ R heteromeric complexes was thus studied by coimmunoprecipitation experiments using a soluble extract from rat cortex, hippocampus and cerebellum membranes (Fig. 3). Coimmunoprecipitation followed by immunoblotting was carried out using anti- A_1 R and anti- $P2Y_1$ R antibodies. Lysates from A_1 R- and $P2Y_1$ R-transfected HEK293T cells showed a lack of cross-reactivity between anti- A_1 R and anti- $P2Y_1$ R antibodies (Fig. 3, lanes 2 and 3 in upper and lower panels), and showed bands at 31–35 kDa and 42–45 kDa corresponding to HA- A_1 R and Myc- $P2Y_1$ R, respectively. Broad bands higher up in lanes 2 and 3 may represent homo-dimers and/or homo-oligomers. When extracts from cortex (lane 5), hippocampus (lane 6) or cerebel-

lum (lane 7) were used as samples, A_1 R (33, 39 kDa) and $P2Y_1$ R (62 kDa) bands were detected in each preparation. It was confirmed that the purified rat brain A_1 R [13] showed the same 39-kDa band by Western blotting (data not shown). The 33-kDa band detected with A_1 R antibody is likely a partially degraded product of A_1 R. Next, coimmunoprecipitation of cell membrane extract from transfected HEK293T cells (lanes 9–11) or rat brain regions (lanes 12–14) was performed. Immunoprecipitation of A_1 R-transfected HEK293T cell extract with anti- A_1 R antibodies followed by Western blotting with anti- A_1 R antibodies revealed major broad bands at 31–35 kDa (upper in Fig. 3, lane 9), corresponding to different glycosylated forms of the HA- A_1 R [3]. Anti- A_1 R antibodies also immunoprecipitated Myc- $P2Y_1$ R in addition to HA- A_1 R from HEK293T cells coexpressing HA- A_1 R and Myc- $P2Y_1$ R (lower in Fig. 3, lane 11). When brain extract from three regions was similarly immunoprecipitated by anti- A_1 R antibodies, a $P2Y_1$ R band (62 kDa) was clearly detected in addition to the A_1 R bands (33, 39 kDa) in every immunoprecipitate (Fig. 3, lanes 12–14). These findings indicate that A_1 R is able to interact with $P2Y_1$ R to form a heteromeric complex in rat cortex, hippocampus and cerebellum.

4. Discussion

Although evidence for the existence of homo- and/or hetero-oligomers of GPCRs with novel functions using a heterologous expression system has been accumulated [14], few studies have dealt with receptor oligomerization in vivo. In fact, our previous report revealed the existence of A_1 R/ $P2Y_1$ R hetero-oligomers only in HEK293T cells cotransfected with the cDNAs for rat A_1 R and $P2Y_1$ R by immunoprecipitation and double immunolabeling experiments [3]. We also demonstrated, by means of bioluminescence resonance energy transfer technology, that A_1 R and $P2Y_1$ R were able to form con-

stitutive hetero-oligomers in intact transfected HEK293T cells and this process was promoted by simultaneous activation of both receptors [8].

In the present paper, double immunostaining and immunoprecipitation experiments using rat brain regions were studied in order to demonstrate the existence of A₁R/P2Y₁R hetero-oligomers *in vivo*. The colocalization of A₁R and P2Y₁R was first analyzed by double immunostaining in primary cultures of neurons from rat cerebral cortex (Fig. 1). A₁R and P2Y₁R were localized and diffusely distributed to the soma and dendrites of the cortical neurons, consistent with previous studies that identified A₁R and P2Y₁R in cell bodies and dendrites [10–12]. The merged images demonstrated the colocalization of these receptors in some parts of the cortical primary neurons. A₁R and P2Y₁R immunoreactivity was successfully shown in the rat cerebral cortex, hippocampus and cerebellum regions and a high degree of A₁R/P2Y₁R colocalization was obtained in these brain sections by the merged images obtained from double immunostaining experiments (Fig. 2). The high degree of colocalization detected by immunohistochemistry of A₁R and P2Y₁R in cerebellar cortex, and other brain sections, supports the notion that A₁R and P2Y₁R may form functional hetero-oligomers in brain.

The existence of A₁R/P2Y₁R hetero-oligomers was then studied in immunoprecipitation experiments by using cell membrane extracts from rat brain (Fig. 3). The antibody against A₁R was able to coimmunoprecipitate P2Y₁R along with A₁R in extracts from the three regions in rat brain. These results indicate that A₁R and P2Y₁R are physically associated, directly or indirectly via an additional component, in brain sections coexpressing A₁R and P2Y₁R, and that these hetero-oligomeric complexes exist constitutively, i.e. in the absence of receptor activation by exogenous agonists.

It is of note that A₁R is able to form homo/hetero-oligomers with several other GPCRs such as A₁R itself [15], dopamine D₁ receptor (D₁R) [16] and metabotropic glutamate_{1α} receptor (mGluR_{1α}) [17], all of which are localized in the central nervous system. The oligomerization process between these receptors seems to be receptor-specific because related subtype receptors do not interact with A₁R [3,16,17], and to be dependent on receptor activation by agonists [8,16]. It is also known that A₁R and A₁R/D₁R complexes interact with adenosine deaminase, a key enzyme for purine metabolism, to induce clustering of adenosine deaminase/A₁R/D₁R complexes [18]. Taken together, these results may suggest a mechanism whereby A₁R is able to form hetero-oligomers with a variety of receptors in the central nervous system, regulated by the distribution and/or activity of receptors to be interacted with, for example, A₁R ↔ A₁R/A₁R ↔ A₁R/P2Y₁R ↔ D₁R/A₁R/P2Y₁R ↔ D₁R/A₁R ↔ D₁R/A₁R/mGluR_{1α} ↔ A₁R/mGluR_{1α} ↔ P2Y₁R/A₁R/mGluR_{1α}.

This study provides the first observation of colocalization of A₁R and P2Y₁R in the rat brain. The significant overlap in A₁R and P2Y₁R expression observed in the same neurons in many brain regions suggests a heteromeric association of both receptors *in vivo*. Coimmunoprecipitation experiments using these brain regions further support this notion. However, it should be noted that a more detailed functional analysis is necessary to prove the physiological roles for the receptor oligomerization in terms of the regulation of purinergic transmissions in the brain.

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