Hetero-oligomerization of adenosine A_1 receptors with $P2Y_1$ 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 A_1 receptor (A_1R) and $G_{q/11}$-coupled $P2Y_1$ receptor ($P2Y_1R$) 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 A_1R and $P2Y_1R$ 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 A_1R with $P2Y_1R$ was shown in coimmunoprecipitation studies using membrane extracts from these regions of rat brain. Together, these results suggest the widespread colocalization of A_1R and $P2Y_1R$ in rat brain, and both receptors can exist in the same neuron, and therefore associate as hetero-oligomeric complexes in the rat brain.**

Key words: Colocalization; Purinergic; Heteromer; Immunohistochemistry

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 A_1, A_{2A}, A_{2B} and A_3 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 ($P2Y_{1,2,4,6,11,12}$), all of which are GPCRs, have been cloned.

The adenosine A_1 receptor (A_1R) 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 $P2Y_1$ receptor ($P2Y_1R$) 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 A_1R and $P2Y_1R$ in HEK293T cells results in the generation of a unique adenosine receptor with P2Y-like agonistic pharmacology via heteromeric complexes between A_1R and $P2Y_1R$ [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 $A_1R/P2Y_1R$ 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 $A_1R/P2Y_1R$ 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 A_1R and $P2Y_1R$ in the same cells. Coimmunoprecipitation experiments were also used to confirm the direct association of A_1R and $P2Y_1R$ 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: A_1R, adenosine A_1 receptor; $P2Y_1R$, $P2Y_1$ receptor; GPCR, G protein-coupled receptor

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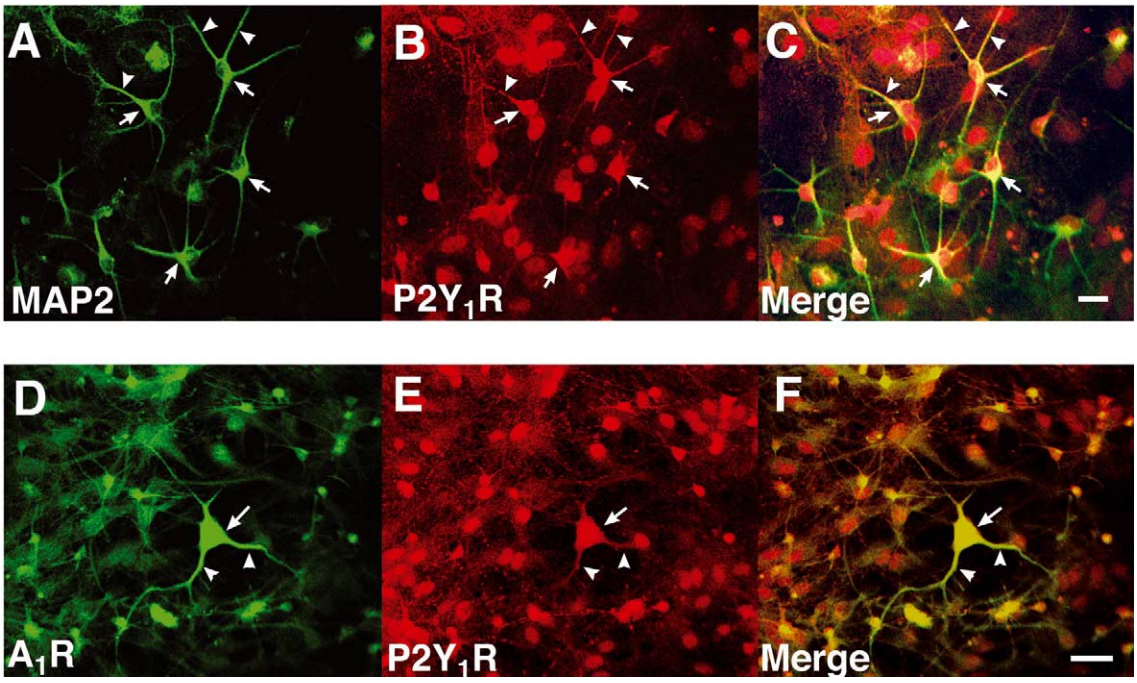

Fig. 1. Double immunofluorescence staining and confocal images of rat cortical cultured neurons. A–C: Staining of MAP2 (A, green image) and $P2Y_1R$ (B, red image) in rat cortical cultured cells, indicating $P2Y_1Rs$ express neural soma and dendrites. Superimposition of images reveals MAP2 and $P2Y_1R$ colocalization in yellow (C). D,E: Staining of A_1R (D, green image) and $P2Y_1R$ (E, red image) in rat cortical cultured cells. Superimposition of images reveals A_1R and $P2Y_1R$ colocalization in yellow (F). The images show a single horizontal optical section of representative cells. Scale bar, 50 μm. 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% CO_2 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-A_1R mouse monoclonal antibody (1 μg/ml) and anti-$P2Y_1R$ rabbit polyclonal antibody (1 μg/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 A_1R, or Alexa 568-conjugated goat anti-rabbit IgG antibody for $P2Y_1R$ (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-μm 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-μm-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-A_1R antibody (1 μg/ml, diluted in blocking solution) and rabbit polyclonal anti-$P2Y_1R$ antibody (1 μg/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-μm 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-A_1R antibody (1 μg/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-A_1R or anti-$P2Y_1R$ 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 A_1R or $P2Y_1R$ cDNA and the immunoprecipitation experiments of the transfected HEK293T cells were performed as described previously [3].

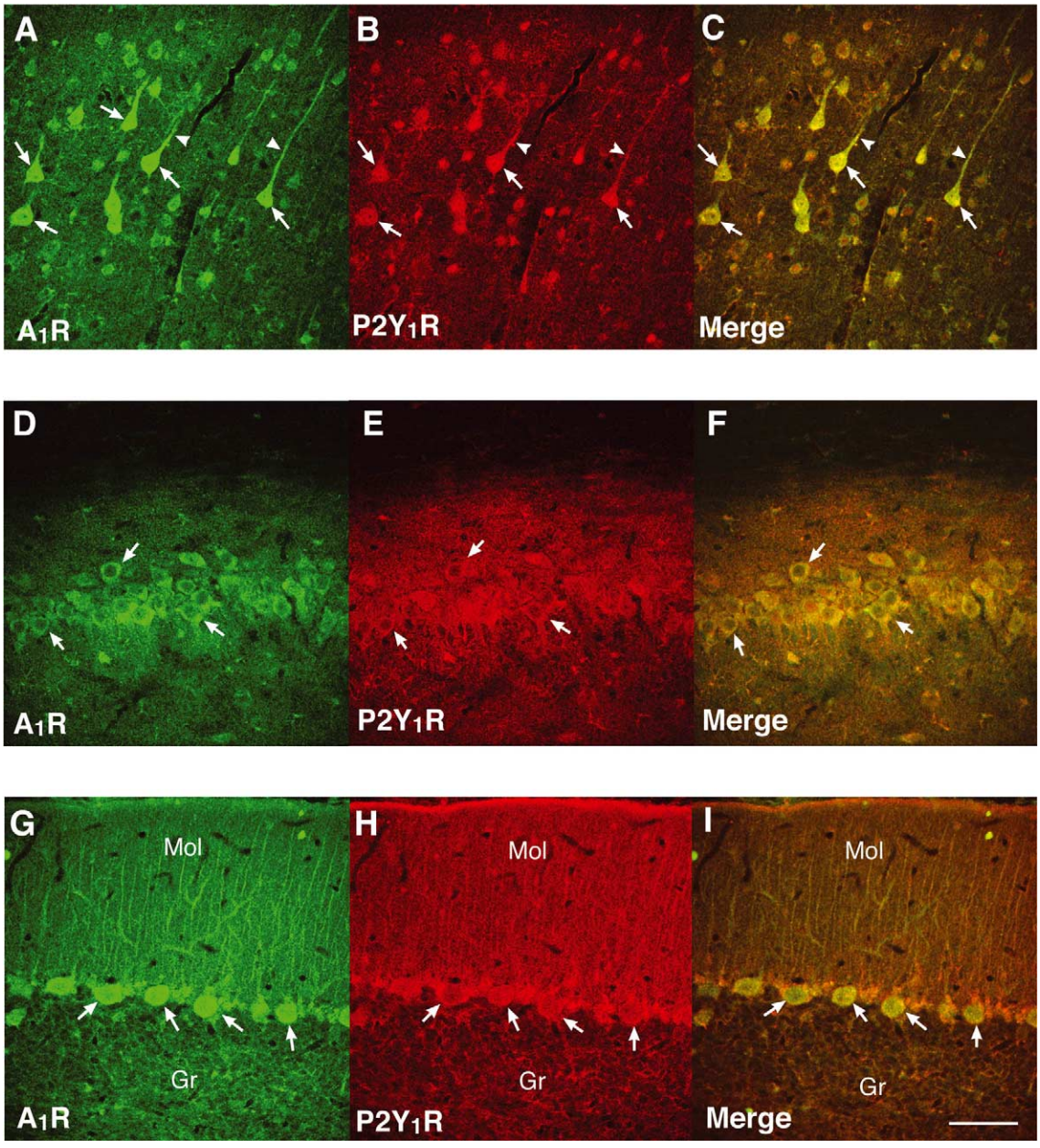

Fig. 2. Double immunofluorescence staining and confocal images of rat brain regions. A_1R and $P2Y_1R$ immunoreactivity was detected in the brain section of cortex (A–C), hippocampus (D–F) and cerebellum (G–I) from rats ($n = 3$–5). Confocal microscopy revealed colocalization (yellow in C, F, I) of A_1R (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]

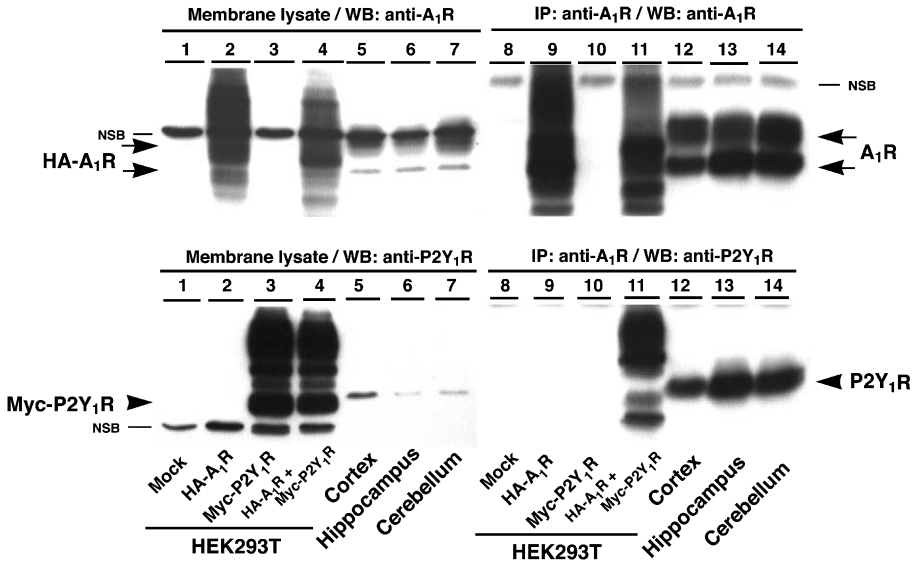

Fig. 3. Coimmunoprecipitation of adenosine A_1Rs and $P2Y_1$Rs from rat cortex, hippocampus and cerebellum. Extracts from various regions were immunoprecipitated with anti-A_1R antibodies, and analyzed by Western blotting with anti-A_1R (upper panels) or anti-$P2Y_1$R (lower panels) antibodies. As a positive control, Myc-$P2Y_1$R was coimmunoprecipitated by anti-A_1R antibodies along with HA-A_1R from the HEK293T cells coexpressing HA-A_1R/Myc-$P2Y_1$R (lane 11). Anti-A_1R antibodies precipitated A_1R (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_1R or HA-A_1R; 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_1R 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_1R 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_1R and $P2Y_1$R immunoreactivity were widely distributed (Fig. 2D,E). Pyramidal cell bodies were significantly stained with both antibodies for A_1R 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_1R 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_1R and $P2Y_1$R over the Purkinje cells and molecular cell layers (yellow in Fig. 2I).

3.2. *Coimmunoprecipitation of A_1R and $P2Y_1$R from rat brain*

The codistribution of A_1R 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_1R/$P2Y_1$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_1R and anti-$P2Y_1$R antibodies. Lysates from A_1R- and $P2Y_1$R-transfected HEK293T cells showed a lack of cross-reactivity between anti-A_1R 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_1R 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 cerebellum (lane 7) were used as samples, A_1R (33, 39 kDa) and $P2Y_1$R (62 kDa) bands were detected in each preparation. It was confirmed that the purified rat brain A_1R [13] showed the same 39-kDa band by Western blotting (data not shown). The 33-kDa band detected with A_1R antibody is likely a partially degraded product of A_1R. 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_1R-transfected HEK293T cell extract with anti-A_1R antibodies followed by Western blotting with anti-A_1R antibodies revealed major broad bands at 31–35 kDa (upper in Fig. 3, lane 9), corresponding to different glycosylated forms of the HA-A_1R [3]. Anti-A_1R antibodies also immunoprecipitated Myc-$P2Y_1$R in addition to HA-A_1R from HEK293T cells coexpressing HA-A_1R and Myc-$P2Y_1$R (lower in Fig. 3, lane 11). When brain extract from three regions was similarly immunoprecipitated by anti-A_1R antibodies, a $P2Y_1$R band (62 kDa) was clearly detected in addition to the A_1R bands (33, 39 kDa) in every immunoprecipitate (Fig. 3, lanes 12–14). These findings indicate that A_1R 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_1R/$P2Y_1$R hetero-oligomers only in HEK293T cells cotransfected with the cDNAs for rat A_1R and $P2Y_1$R by immunoprecipitation and double immunolabeling experiments [3]. We also demonstrated, by means of bioluminescence resonance energy transfer technology, that A_1R 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 $A_1R/P2Y_1R$ hetero-oligomers in vivo. The colocalization of A_1R and $P2Y_1R$ was first analyzed by double immunostaining in primary cultures of neurons from rat cerebral cortex (Fig. 1). A_1R and $P2Y_1R$ were localized and diffusely distributed to the soma and dendrites of the cortical neurons, consistent with previous studies that identified A_1R and $P2Y_1R$ in cell bodies and dendrites [10–12]. The merged images demonstrated the colocalization of these receptors in some parts of the cortical primary neurons. A_1R and $P2Y_1R$ immunoreactivity was successfully shown in the rat cerebral cortex, hippocampus and cerebellum regions and a high degree of $A_1R/P2Y_1R$ 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 A_1R and $P2Y_1R$ in cerebellar cortex, and other brain sections, supports the notion that A_1R and $P2Y_1R$ may form functional hetero-oligomers in brain.

The existence of $A_1R/P2Y_1R$ hetero-oligomers was then studied in immunoprecipitation experiments by using cell membrane extracts from rat brain (Fig. 3). The antibody against A_1R was able to coimmunoprecipitate $P2Y_1R$ along with A_1R in extracts from the three regions in rat brain. These results indicate that A_1R and $P2Y_1R$ are physically associated, directly or indirectly via an additional component, in brain sections coexpressing A_1R and $P2Y_1R$, and that these hetero-oligomeric complexes exist constitutively, i.e. in the absence of receptor activation by exogenous agonists.

It is of note that A_1R is able to form homo/hetero-oligomers with several other GPCRs such as A_1R itself [15], dopamine D_1 receptor (D_1R) [16] and metabotropic glutamate$_{1\alpha}$ receptor ($mGluR_{1\alpha}$) [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 A_1R [3,16,17], and to be dependent on receptor activation by agonists [8,16]. It is also known that A_1R and A_1R/D_1R complexes interact with adenosine deaminase, a key enzyme for purine metabolism, to induce clustering of adenosine deaminase/A_1R/D_1R complexes [18]. Taken together, these results may suggest a mechanism whereby A_1R 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, $A_1R \leftrightarrow A_1R/A_1R \leftrightarrow A_1R/P2Y_1R \leftrightarrow D_1R/A_1R/P2Y_1R \leftrightarrow D_1R/A_1R \leftrightarrow D_1R/A_1R/mGluR_{1\alpha} \leftrightarrow A_1R/mGluR_{1\alpha} \leftrightarrow P2Y_1R/A_1R/mGluR_{1\alpha}$.

This study provides the first observation of colocalization of A_1R and $P2Y_1R$ in the rat brain. The significant overlap in A_1R and $P2Y_1R$ 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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