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# ATP- and Adenosine-Mediated Signaling in the Central Nervous System: Purinergic Receptor Complex: Generating Adenine Nucleotide-Sensitive Adenosine Receptors

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**Abstract.** Adenosine $A_1$ receptors ($A_1R$) are able to form a heteromeric complex with $P2Y_1$ receptors ($P2Y_1R$) that generates $A_1R$ with $P2Y_1R$-like agonistic pharmacology. A potent $P2Y_1R$ agonist, adenosine 5'-*O*-(2-thiotriphosphate), binds the $A_1R$ binding pocket of the $A_1R/P2Y_1R$ complex and inhibits adenylyl cyclase activity via $G_{i/o}$ protein. These mechanisms might be used to fine-tune purinergic inhibition locally at sites where there is a particular oligomerization structure between purinergic receptors and explain the undefined purinergic functions by adenosine and adenine nucleotides.

***Keywords***: oligomerization, purinergic receptor, bioluminescence resonance energy transfer system (BRET), immunoprecipitation, G protein-coupled receptor

## Introduction

To date, 12 subtypes of the trimeric G protein-coupled purinergic receptor family have been discovered, including four types of P1 receptors and eight types of P2Y receptors (1). The adenosine $A_1$ receptor ($A_1R$) is functionally coupled to pertussis toxin (PTX)-sensitive $G_{i/o}$ proteins, and its activation modulates several effectors: inhibition of adenylyl cyclase, activation of $K^+$ channels, and inhibition of $Ca^{2+}$ channels; the latter two would inhibit neuronal activity. On the other hand, the $P2Y_1$ receptor ($P2Y_1R$) stimulates phospholipase C$\beta$ through PTX-insensitive G protein, $G_{q/11}$, leading to the formation of diacylglycerol as well as inositol trisphosphate ($IP_3$) and mobilization of intracellular $Ca^{2+}$. Recently, several studies suggest that not only adenosine but also ATP can inhibit synaptic transmission (2 – 7). It is easily speculated that adenosine produced from ATP breakdown acts on presynaptic $A_1R$ to inhibit transmitter release. However, several reports indicated nucleotides per se can inhibit transmission and conversion to adenosine by endogenous ecto-nucleotidases is not required (4, 5). These results raise the possibility of a unique nucleotide receptor that is activated directly by ATP and ATP analogues and is insensitive to most P2 receptor antagonists, but is sensitive to selective $A_1R$ antagonists, and is able to inhibit neurotransmitter release like adenosine itself (4, 5, 8, 9). However, identification of such ATP-responsive receptors has not been achieved yet.

Recently, a significant amount of G protein-coupled receptor (GPCR) has been reported to exist in a homomeric and heteromeric assembly to change their pharmacology or function (10). We therefore predicted that, like other GPCRs, P1 receptors can potentially form heteromeric complexes with distinct types of GPCRs through direct association. In fact, previous radioligand binding and biochemical studies (11, 12) indicated that $A_1R$ could be arranged in dimeric complexes with related proteins or GPCRs. However, to date, no evidence of direct interaction between G protein-coupled P1 and P2 receptors that induces functional changes in cells or tissues has been obtained, although functional interactions between $A_1R$ and P2YR has been previously described (13). Previous studies showed that $P2Y_1R$

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localized in neuronal cells of the hippocampus, midbrain, and subthalamic nucleus and associated regions (14) and that $A_1R$ localized in the cerebral cortex, hippocampus, and thalamus, especially in the neuronal cells of these regions (15). Therefore, a significant portion of $A_1R$ and $P2Y_1R$ distributed in the central nervous system is likely to co-localize in the overlap regions and thereby exert new functions. The purpose of this study is to determine whether P1 and P2 receptors, in this case $A_1R$ and $P2Y_1R$, can form a hetero-oligomer that exerts novel pharmacological and functional characteristics with a potential role in the purinergic-signaling cascade.

In this article, we describe our recent progress in the demonstration of hetero-oligomerization between $A_1R$ and $P2Y_1R$, an actual hybrid purinergic receptor that enables the hypothesized nucleotide receptor to respond to adenosine, with a potential role in the purinergic signaling cascade using a recombinant receptor technology in cultured cell systems.

## Heteromeric association of $A_1R$ and $P2Y_1R$ in cotransfected cells

We transiently cotransfected hemagglutinin (HA)-tagged $A_1$ receptor (HA-$A_1R$) and Myc-tagged $P2Y_1$ receptor (Myc-$P2Y_1R$) cDNA into HEK293T cells and observed whether $A_1R$ and $P2Y_1R$ associate with each other as a heteromeric complex by conducting immunoprecipitation experiments using whole-cell membrane lysates. As shown in Fig. 1, anti-HA antibody precipitated both Myc-$P2Y_1R$ and HA-$A_1R$ from cotransfected cells. However, it did not precipitate Myc-dopamine $D_2$ receptor ($D_2R$) along with HA-$A_1R$. Conversely, anti-Myc antibody immunoprecipitated HA-$A_1R$ along with Myc-$P2Y_1R$ (16). Such counterimmunoprecipitation was not observed with the admixture of cell membranes expressing each receptor individually (K. Yoshioka et al., unpublished data). The C-tail deletion mutant of $A_1R$ was still able to associate with $P2Y_1R$ in HEK293T cells (K. Yoshioka et al., unpublished data), suggesting that the C-tail of $A_1R$ is not required for the heteromeric association with $P2Y_1$ receptors. The appearance of additional bands is likely due to the heterogeneity of glycosylation of the receptors, because most bands shifted to a position corresponding to a low molecular mass band following treatment with *N*-glycosidase F (16). These results indicate that $A_1R$ is able to form heteromeric complexes with either $P2Y_1R$ or $P2Y_2R$ when transfected simultaneously in HEK293T cells.

## Coexpression with $P2Y_1R$ modulates $A_1R$ binding pharmacology and functional G protein-coupling in cotransfected cells

We examined ligand-binding pharmacology of the cotransfected cell membranes using P1 receptor agonist [$^3$H]NECA (5'-*N*-ethylcarboxamidoadenosine) by competition experiments. The apparent binding potency and efficacy of the $A_1R$ selective agonist CPA ($N^6$-cyclopentyladenosine) to the [$^3$H]NECA binding site were reduced in the co-transfected cells (Fig. 2A, left). The selective $P2Y_1R$ antagonist MRS2179 ($N^6$-methyl-2'-deoxyadenosine-3',5'-bisphosphate) failed to displace [$^3$H]NECA bound to HA-$A_1R$-transfected and HA-$A_1R$/Myc-$P2Y_1R$-transfected cell membranes (data not shown). A potent $P2Y_1R$ agonist ADP$\beta$S (adenosine 5'-*O*-(2-thiotriphosphate)) was found to displace the ligand from the [$^3$H]NECA binding site of co-transfected cell membranes with $K_i$ values of $0.38 \pm 0.05$ nM (high-affinity site) and $610 \pm 85$ nM (low-affinity site). In contrast, ADP$\beta$S in the $10^{-6}$ M range slightly inhibited [$^3$H]NECA binding of cell membranes expressing HA-$A_1R$ alone ($K_i = 1670 \pm 98$ nM, Fig. 2A, right).

We next studied $A_1R$ agonist-induced adenylyl cyclase

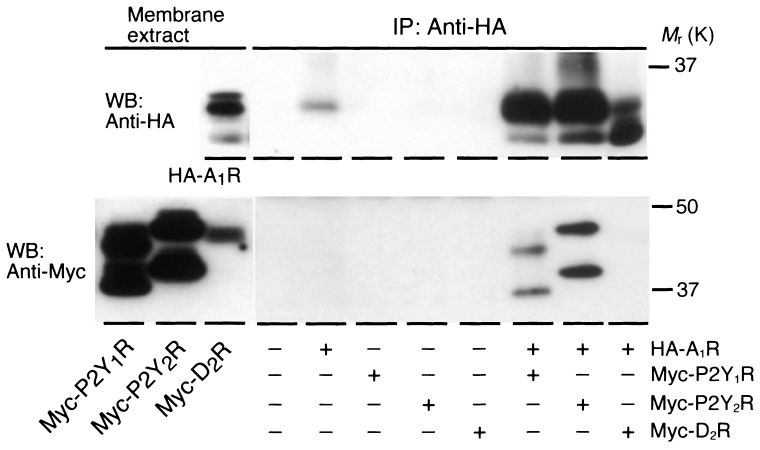


**Fig. 1.** Association of $A_1R$ and $P2Y_1R$ or $P2Y_2R$, but not $D_2R$, in cotransfected HEK293T cells. Coimmunoprecipitation of cell lysates by anti-HA antibody was performed followed by Western blots with anti-HA (upper) and anti-Myc (lower) antibodies. In addition to HA-$A_1R$ (35 and 31 kDa), anti-HA antibody coimmunoprecipitated Myc-$P2Y_1R$ (42 and 37 kDa) from the cell membrane lysates coexpressing HA-$A_1R$/Myc-$P2Y_1R$ (lower: lane 9 from the left). Myc-$P2Y_2R$ (39 and 45 kDa) also was coimmunoprecipitated by anti-HA antibody along with HA-$A_1R$ from the cell lysates coexpressing HA-$A_1R$/Myc-$P2Y_2R$ (lower: lane 10 from the left). In contrast, Myc-$D_2R$ was not immunoprecipitated from the cell lysates coexpressing HA-$A_1R$/Myc-$D_2R$ (lower: lane 11 from the left) by anti-HA antibody. Data are representative of 2 – 4 independent experiments. Modified from ref. 16.

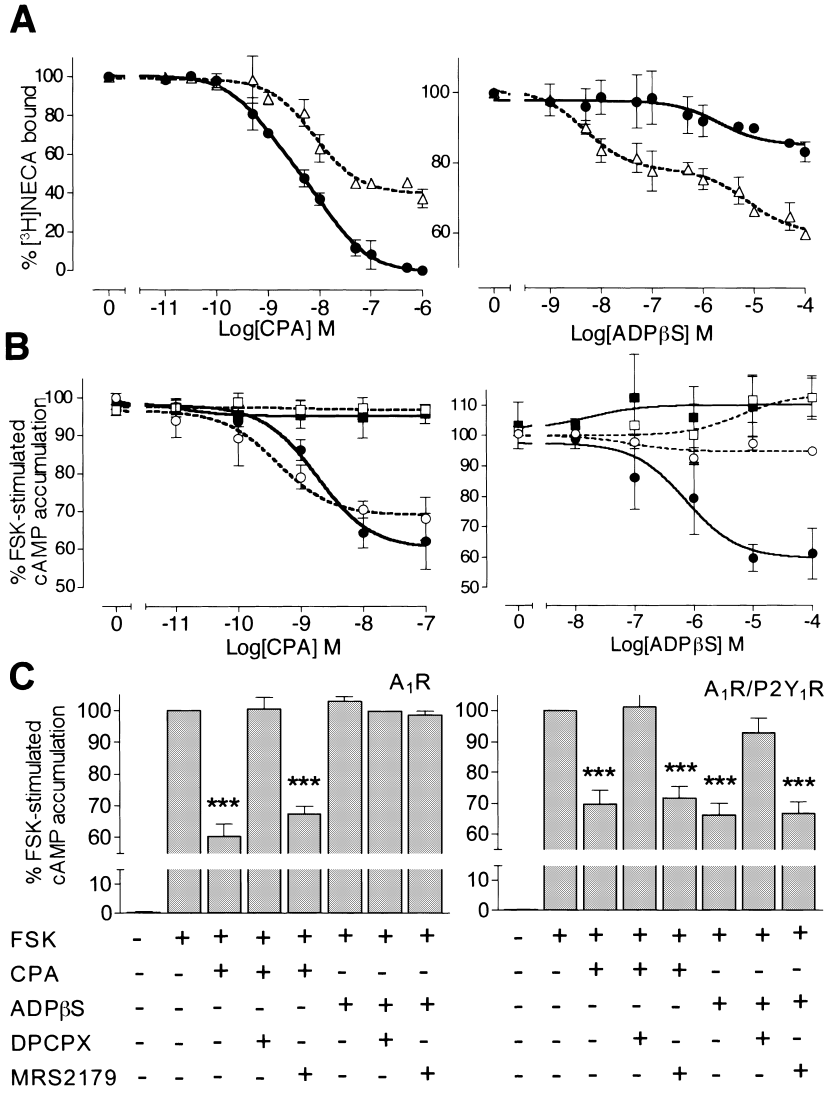


**Fig. 2.** Coexpression with $P2Y_1R$ modulates $A_1R$ binding pharmacology and generates $P2Y_1R$ agonist-sensitive adenylyl cyclase inhibition of $A_1R$. A) Displacement of [$^3$H]NECA (40 nM) binding with transfected cell membranes by CPA (left) and ADP$\beta$S (right). Membranes from HA-$A_1R$-transfected (closed circle) or HA-$A_1R$/$P2Y_1R$-transfected (open triangle) cells were incubated with the indicated concentrations of each ligand. The [$^3$H]NECA concentrations were selected to ensure maximal saturation binding. Data represent the means ± S.E.M. of the percentage of [$^3$H]NECA specific bound values. Results from 3 independent experiments performed in duplicate are shown. B) Concentration-dependent reduction of maximal FSK (10 $\mu$M)-stimulated intracellular cAMP accumulation by CPA (left) or ADP$\beta$S (right) in $A_1R$/$P2Y_1R$-transfected cells. Dotted line, cells expressed HA-$A_1R$ alone; solid line, cells co-expressed HA-$A_1R$ and Myc-$P2Y_1R$; circle, non-treated cells; square, PTX-pretreated cells (100 ng/ml, 16 h). The 100% values of cAMP for the cells transfected with HA-$A_1R$, and HA-$A_1R$ plus Myc-$P2Y_1R$ were 72 ± 14 and 67 ± 19 pmol/$10^5$ cells, respectively (mean ± S.E.M., n = 5). C) Pretreatment of cells with the $A_1R$ antagonist DPCPX, but not the $P2Y_1R$ antagonist MRS2179, significantly inhibited maximal ADP$\beta$S-induced adenylyl cyclase attenuation in the $A_1R$/$P2Y_1R$-transfected cells. Left, HA-$A_1R$ transfected cells; right, HA-$A_1R$/Myc-$P2Y_1R$ co-transfected cells. The 100% values of cAMP for the cells transfected with HA-$A_1R$, and HA-$A_1R$/Myc-$P2Y_1R$ were 70 ± 12 and 71 ± 17 pmol/$10^5$ cells, respectively (mean ± S.E.M., n = 5). Data represent the means ± S.E.M. of the percentage of FSK-induced cAMP accumulation values. Results from 3 to 5 independent experiments performed in duplicate are shown. ***$P$<0.01, Student's $t$-test. Modified from ref. 16.

inhibition, a main index of $A_1R$ function, in co-transfected cells (Fig. 2B). The cells expressing $A_1R$ alone revealed an inhibition of forskolin (FSK)-stimulated cAMP accumulation by CPA in a dose-dependent manner, with the estimated concentration for half-maximal response ($IC_{50}$) of 0.42 ± 0.1 nM to a maximum inhibition of 70 ± 6%. This activity was completely abolished by pretreatment of the cells with PTX. CPA-induced inhibition of FSK-stimulated adenylyl cyclase activity was also detected with the estimated $IC_{50}$ value of 1.0 ± 0.12 nM in the cells co-expressing $A_1R$/$P2Y_1R$. This activity was also abolished by PTX treatment (Fig. 2B, left). The potency of adenylyl cyclase attenuation by CPA was reduced significantly in the co-expressing cells compared with cells expressing $A_1R$ alone ($P$<0.05, Student $t$-test). The treatment of cells expressing $A_1R$ alone with ADP$\beta$S revealed no changes in FSK-stimulated cAMP production (Fig. 2B, right). Activation of $P2Y_1R$-transfected cells with ADP$\beta$S did not lead to a significant change in FSK-evoked cAMP levels (data not shown). In cells co-expressed with $A_1R$ and $P2Y_1R$, ADP$\beta$S markedly reduced FSK-evoked adenylyl cyclase activity in a concentration-dependent manner, with the estimated $IC_{50}$ value of 730 ± 35 nM, to a maximum inhibition of 62 ± 9%. PTX treatment resulted in complete loss of the dose-dependent activity of ADP$\beta$S, suggesting the involvement of a PTX-sensitive $G_{i/o}$ protein (Fig. 2B, right). We examined whether the ADP$\beta$S-induced adenylyl cyclase inhibition in co-expressed cells was mediated through the ligand-binding site of $A_1R$ (Fig. 2C). In both $A_1R$-expressing cells and $A_1R$/$P2Y_1R$-coexpressing cells, CPA (10 nM) maximally inhibited the FSK-evoked adenylyl cyclase activity to virtually identical extents. This inhibitory effect was blocked in the presence of $A_1R$ antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine). When cells co-expressing $A_1R$

/P2Y$_1$R were pretreated with DPCPX, however, the ADP$\beta$S-evoked adenylyl cyclase inhibition was decreased by approximately 95%, whereas MRS2179 had no effect on the ADP$\beta$S-evoked adenylyl cyclase inhibition. Taken together, these results suggest that ADP$\beta$S exerts the adenylyl cyclase inhibitory activity through xanthine-sensitive ligand-binding sites of A$_1$R via a G$_{i/o}$ protein-linked effector system.

### Agonist-promoted hetero-oligomerization of A$_1$R and P2Y$_1$R in living cells

Although an increasing number of reports describing the existence and functional importance of homo- and hetero-oligomerization of GPCRs has appeared (10), the mechanism of the oligomerization is still largely unknown, and it is not always clear whether such oligomers persist in living cells and whether the binding of agonist regulates oligomerization. To answer these questions, co-immunoprecipitation strategies seem limited because the solubilization of hydrophobic proteins such as GPCRs can cause artifactual aggregations or the solubilization process itself can inhibit the association between GPCRs. To determine whether there is a constitutive association between A$_1$R and P2Y$_1$R in living cells, we measured this interaction with an improved bioluminescence resonance energy transfer system (BRET$^2$) (offered by PerkinElmer Life Sciences, Yokohama) (17) in HEK293T cells co-transfected with either HA-A$_1$R-GFP$^2$ (HA-A$_1$R fused to modified green fluorescent protein) / HA-A$_1$R-Rluc (HA-A$_1$R fused to Renilla luciferase) or HA-A$_1$R-GFP$^2$ / Myc-P2Y$_1$R-Rluc (Myc-P2Y$_1$R fused to Rluc) (18). As shown in Fig. 3A, co-expression of HA-A$_1$R-GFP$^2$/HA-A$_1$R-Rluc (BRET ratio = 0.062 ± 0.004, n = 15) or HA-A$_1$R-GFP$^2$ /Myc-P2Y$_1$R-Rluc (BRET ratio = 0.07 ± 0.008, n = 20) upon addition of Rluc substrates resulted in a small but significant increase in the BRET ratio ($P$<0.05 *vs* control cells) under basal conditions. Co-expression of the isolated Rluc along with HA-A$_1$R-GFP$^2$ resulted in a weak energy transfer (BRET ratio = 0.045 ± 0.005, n = 6), indicating that there was no direct interaction between these two constructs (Fig. 3A, lower line). Similarly, co-expression of isolated GFP$^2$ with Myc-P2Y$_1$R-Rluc failed to produce a significant energy transfer signal (BRET ratio = 0.048 ± 0.006, n = 6) (data not shown). These results provide strong evidence of an actual association between either A$_1$R-GFP$^2$ and A$_1$R-Rluc or A$_1$R-GFP$^2$ and P2Y$_1$R-Rluc in intact cells. The extent of the heteromeric association is substantially greater than that of the homomeric association of A$_1$R ($P$<0.05). Incubation of HA-A$_1$R-GFP$^2$/Myc-P2Y$_1$R-Rluc-co-transfected cells with the agonists CPA and ADP$\beta$S

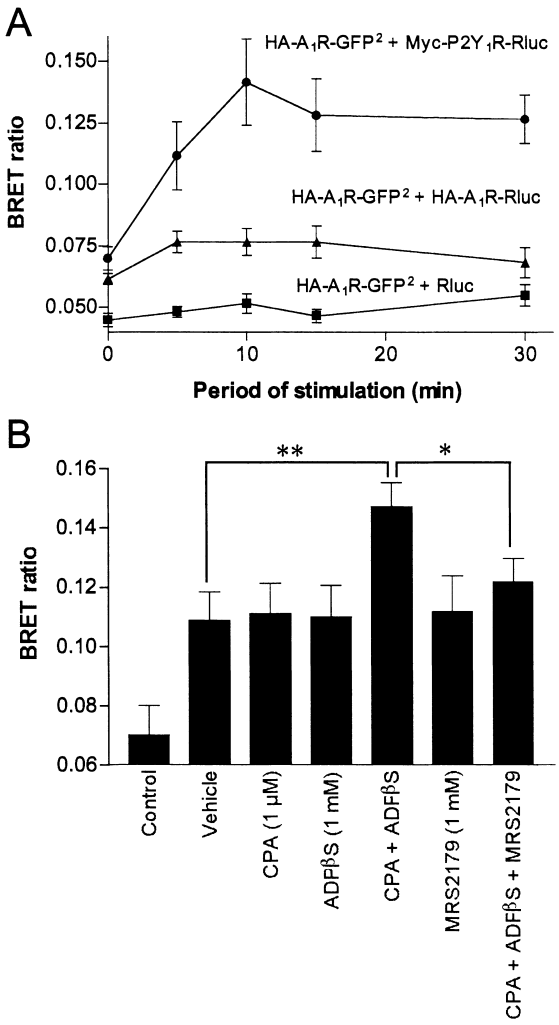


**Fig. 3.** BRET$^2$ detection of constitutive and agonist-promoted oligomerization of HA-A$_1$R-GFP$^2$ and Myc-P2Y$_1$R-Rluc in living HEK293T cells. A) Time-dependent BRET$^2$ signal in living HEK293T cells co-expressing HA-A$_1$R-GFP$^2$ and HA-A$_1$R-Rluc (homo-oligomer, triangle), HA-A$_1$R-GFP$^2$ and Myc-P2Y$_1$R-Rluc (hetero-oligomer, circle), or HA-A$_1$R-GFP$^2$ and Rluc (control, square). Cells were incubated with agonists of A$_1$R and P2Y$_1$R (1 $\mu$M CPA + 100 $\mu$M ADP$\beta$S) before the addition of Rluc substrates. The data shown represent the mean ± S.E.M. of three independent experiments performed in triplicate for each time point. B) BRET$^2$ ratio was measured in HEK293T cells co-transfected with HA-A$_1$R-GFP$^2$ and Myc-P2Y$_1$R-Rluc. Cells were incubated with either CPA (1 $\mu$M), ADP$\beta$S (100 $\mu$M), P2Y$_1$R antagonist MRS2179 (1 mM) or a combination thereof for 10 min before the addition of Rluc substrate. The data represent the mean ± S.E.M. of three independent experiments; **$P$<0.01, compared with vehicle treatment and *$P$<0.05, compared with CPA and ADP$\beta$S treatment. Modified from ref. 18.

increased the BRET ratio with a maximum being reached at 10 min (Fig. 3A, upper line). The agonist-promoted BRET signal observed between HA-A$_1$R-GFP$^2$ and Myc-P2Y$_1$R-Rluc did not result from nonspecific association between the GFP$^2$ and Rluc proteins since

no increase in the signal intensity was detected in cells expressing either HA-$A_1$R-GFP$^2$/Rluc or HA-$A_1$R-GFP$^2$/HA-$A_1$R-Rluc as shown above. It was also confirmed that the BRET signal did not strengthen in cells co-expressing GFP$^2$ and Rluc ($0.045 \pm 0.008$, n = 6). Incubation of HA-$A_1$R-GFP$^2$/HA-$A_1$R-Rluc expressing cells with agonists did not result in a significant increase in the BRET signal (Fig. 3A, middle line).

To demonstrate the specificity of the agonist-dependent increase in the BRET ratio, HA-$A_1$R-GFP$^2$/Myc-$P2Y_1$R-Rluc-transfected cells were incubated for 10 min in the presence of several ligands (Fig. 3B). A significant increase in the ratio was again observed in the presence of both agonists, but not with either alone. This increase was significantly inhibited by pretreatment with MRS2179, a potent $P2Y_1$R antagonist, although the addition of MRS2179 alone had no effect on the BRET ratio.

Human $\beta_2$-adrenergic receptors are similarly reported to form constitutive and agonist-dependent homodimers when expressed in HEK293 cells as determined by traditional BRET (19). It has been shown in immunoprecipitation experiments that $A_1$R/$D_1$R hetero-oligomerization in co-transfected fibroblast cells disappeared on pretreatment with a $D_1$R agonist, but not combined pretreatment with a $D_1$R and $A_1$R agonists (11). McVey et al. (20) have reported that the hetero-oligomer of $\delta$-opioid receptor and $\beta_2$-adrenoreceptor accumulated in the presence of an agonist for either receptor. In contrast, using a similar BRET technique, it was found that the human $\delta$-opioid receptor forms homo-oligomers constitutively that was not further regulated by ligand occupancy. These observations suggest that the role of agonist-occupancy of receptors in homo- or hetero-oligomerization of GPCRs can differ between receptors. The present result in the BRET$^2$ experiment that combined agonists further promoted hetero-oligomerization of two purinergic receptors may support this observation.

## Co-localization and association of $A_1$R and $P2Y_1$R in rat brain

Immunohistochemical studies showed that immunoreactivity for both $A_1$R and $P2Y_1$R is present in the cortex, hippocampus and cerebellum (21). 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 (14, 15).

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 demonstrated by coimmunoprecipitation experiments using a soluble extract from rat cortex, hippocampus, and cerebellum membranes (21). Coimmunoprecipitation followed by immunoblotting was carried out using anti-$A_1$R and anti-$P2Y_1$R antibodies. 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. 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 (21). 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.

## Future perspectives on the purinergic receptor complex

Our findings suggest a mechanism whereby $A_1$R is able to form a heteromeric complex with $P2Y_1$R in the central nervous system, and this complex formation is regulated by the distribution and/or activity of receptors. One of the physiological relevances of $A_1$R/$P2Y_1$R hetero-oligomerization to neural functions may be a quick attenuation of transmitter release by ATP, rather than that by adenosine, if ATP itself works as an $A_1$R agonist to inhibit transmission. In terms of regulation of neurotransmission by adenine nucleotides, several studies indicate the presence of atypical subtypes of P2YRs (3, 5, 22 – 24) that are sensitive to the P1-receptor antagonist theophylline or PTX. Although the molecular basis for these observations has not been well described, these atypical P2YRs are suggested to be responsible for ATP-dependent inhibition of synaptic transmission that is distinct from the usual adenosine response (4, 5). More recently, Masino et al. (7) reported similar inhibitory effect of ATP or ATP$\beta$S on hippocampal glutamatergic transmission that is dependent on $A_1$R. Three possibilities for the ATP-response were raised in the article: 1) nucleotides bind to and directly activate $A_1$R; 2) ectonucleotidases metabolize extracellular nucleotides to adenosine, which then activates $A_1$R; 3) nucleotides activate novel ATP receptors antagonized by classical $A_1$R antagonists such as CPT. The experimental data supported the second possibility because ATP$\beta$S was a weak displacer of $A_1$R binding and was metabolized significantly to adenosine during the incubation. The result that the ATP response is lost in the $A_1R^{-/-}$ mice did not support the third possibility. It is thus very interesting to compare the properties of our $A_1$R/$P2Y_1$R hetero-oligomers described in the

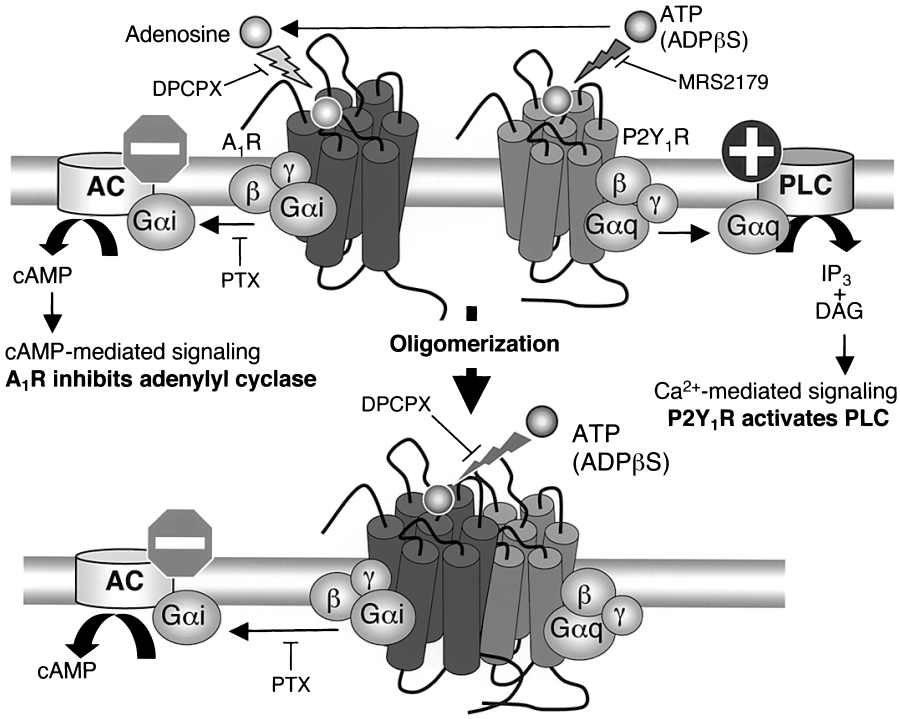


**Fig. 4.** Assumptive scheme for the heteromeric association of $A_1R$ and $P2Y_1R$. After the heteromeric association of $A_1R$ and $P2Y_1R$, a potent $P2Y_1R$ agonist, ADP$\beta$S, can bind with an $A_1R$ binding pocket that is xanthine-sensitive and inhibits adenylyl cyclase activity via the $G_{i/o}$ protein-linked effector system. AC, adenylyl cyclase; PLC, phospholipase C; DAG, diacylglycerol; see text for additional abbreviations.

present study with the atypical ATP-dependent properties reported from other laboratories. It is evident that hetero-oligomerization alters the pharmacology of $A_1R$ to ATP-responsible $A_1R$ in both ligand binding and adenylate cyclase coupling. These new functions obtained in the heteromers are all $A_1R$ antagonist-sensitive and PTX-sensitive. The possible signaling scheme is illustrated in Fig. 4. Therefore, the general pharmacology of $A_1R/P2Y_1R$ heteromers seems very similar to that of the atypical ATP response described above, although existence of functional hetero-oligomers between $A_1R$ and P2YR in intact brain including hippocampus that is sensitive to PTX should be determined.

Thus, heteromeric association between purinergic receptors that generate hybrid pharmacology, that is, adenine nucleotide-sensitive adenosine receptors, may be one of the clues to undefined diverse physiological functions of ATP in various tissues and cells.

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