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Application of Quantitative Immunogold Electron Microscopy to Determine the Distribution and Relative Expression of Homo- and Heteromeric Purinergic Adenosine A1 and P2Y Receptors

Kazunori Namba
National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo Japan

1. Introduction

The idea that G-protein-coupled receptors (GPCRs) may generate or modify various functions as dimmers or higher-order oligomers is now generally accepted. Significant numbers of GPCRs exit as heteromeric assemblies (refered to as hetero-oligomerization), generating novel functions for ligand binding and second messengers, and in turn creating unique receptor trafficking systems for pharmacological profiles (Angers *et al*., 2002, Bulenger *et al*., 2005). This is also true of the purinergic receptor family. Over recent years, we have explored many biochemical and pharmacological aspects of this particular family via hetero-oligomerization between metabotropic (i.e. G protein-coupled) purinergic receptors (particularly between P1 and P2), in which the agonists are metabolites playing important role in the purinergic signaling cascade.

Purines such as adenosine triphosphate (ATP), via their specific P1 and P2 receptors, mediate a variety of physiological processes including pathophysiology, neurotransmission, neuromodulation, pain, cardiac function, immune responses and almost every aspect of development (Abbracchio *et al*., 2009; Burnstock, 2007; Burnstock, 2008; Ralevic *et al*., 1998). P1 receptors are further sub-classified into A_1, A_{2A}, A_{2B} and A_3 sub-types, all of which are G protein-coupled receptors (GPCRs). The adenosine A_1 receptor (A_1R) is known to regulate Ca^{2+}/K^+ channels, adenylate cyclase, and phospholipase C by coupling to $G_{i/o}$ proteins (Ralevic *et al*., 1998). The P2 receptors can be further sub-classified into ligand-gated ion channel-type $P2X_{(1\text{-}7)}$ receptors, and G protein-coupled $P2Y_{(1, 2, 4, 6, 11, 12, 13, 14)}$ receptors. $P2Y_2R$-specific pharmacology (induction of Ca^{2+} release) has been analyzed in detail using CHO-K1 cells (Mehta *et al*., 2008). In hippocampal astrocytes, $P2Y_1R$- and $P2Y_2R$-mediated Ca^{2+} responses differentially show two forms of activity-dependent negative feedback of synaptic transmission via the phospholipase C beta-IP^3 pathway (Fam *et al*., 2003). $P2Y_2$ R modulation of pain responses has also been reported (Molliver *et al*., 2002). Today, homo- or hetero-oligomers of many kinds of GPCRs have been reported (Bouvier, 2001) and the hetero-oligomerization of GPCRs affects various aspects of receptor function, including the alteration of ligand-binding specificity and cellular trafficking. We previously demonstrated

that A_1R associates with $P2Y_1R$ in co-transfected HEK293T cells and in rat brain homogenates, whereby a $P2Y_1R$ agonist stimulates A_1R signaling via $G_{i/o}$ (Yoshioka *et al*., 2001, Yoshioka *et al*., 2002). Furthermore, in co-transfected HEK293T cells, hetero-oligomers display unique pharmacology whereby simultaneous activation of the two receptors attenuates A_1R signaling via $G_{i/o}$, but synergistically enhances $P2Y_2R$ signaling via $G_{q/11}$ (Suzuki *et al*., 2006). Because A_1R are widely expressed in the brain (Yoshioka *et al*., 2002), it is likely that these receptors also associate directly *in situ*; however, direct evidence of their oligomerization or precise co-localization in brain has yet to be demonstrated. In our laboratory, we are developing a new method, immunogold electron microscopic observation using different sized-immunogold particles enable visualize the oligomerization of A_1R and $P2Y_2R$ (Namba *et al*., 2010). The aim of the study was to determine whether A_1R and $P2Y_2R$ associate with each other in the rat brain by looking for receptor complexes with immunogold electron microscopy (IEM). This method also provides information concerning the localization and density of GPCR monomers and oligomers expressed in transfected cells, that are also applicable to tissues such as brain.

In this chapter, we describe both pre- and post-embedding electronmicroscopic techniques to identify cells or tissues expressing GPCRs utilizing differently-sized immunogold particles, and review IEM quantification as an efficient approach to analyze two specific types of data. One data set represents the classification of receptor formations. A_1R and $P2Y_1R$ ($P2Y_2R$) produce five receptor formations which are made up of monomers (A_1R, P2YR), homo-oligomers (A_1R- A_1R, P2YR- P2YR) and hetero-oligomers (A_1R-P2YR). The second dataset describes the estimation of receptor expression levels by counting immunoreactive immunogold particles at the cell surface.

Establishing specific expression patterns of GPCRs at the ultrastructural level, and detecting homo- and hetero-oligomers of GPCRs in both co-transfected cultured cells and tissues, will enable us to visually understand some of the phenomena underlying signal transduction signalling pathways operating via GPCRs in a heteromeric dependent manner. It is widely accepted that drug discovery targets for rapid remedies are likely to be specific receptors expressed upon the cytoplasmic membrane. In order to establish the precise effects of new drugs, the expression patterns and expression level of A_1R and $P2Y_1R$ ($P2Y_2R$) represent significant factors to be considered, especially with regard to their association with cross-talk systems.

2. Immunostaining of GPCRs in transfected HEK293T cells and brain sections

Double immunofluororescence microscopic methods is now generally employed for studying the co-localization of GPCRs in transfected cells. Transient transfection using HEK293T cells with epitope tagged-receptors (Hemagglutinin: HA- or Myc-) in expression plasmids has been performed routinely in our laboratory (Yoshioka *et al*., 2002; Nakata *et al*., 2006). Before commencing immunogold electron microscopy, we routinely analyse, the subcelluar distribution of HA-A_1R and Myc-$P2Y_1R$ ($P2Y_2R$) in co-transfected cells by immunocytochemistry and confocal laser microscopy (Yoshioka *et al*., 2001; Namba *et al*., 2010). Confocal imaging of co-localized GPCRs provides highly detailed information regarding their co-localization upon cellular organelles, an important feature for the subsequent analysis of co-localization in ultrastructural images obtained by transmission electronmicroscopy. If two genes are co-localized at specific cellular organelles, then there is

a much higher probability of hetero-oligomerization. Thus, confocal images of co-localization between A_1R and $P2Y_1R$ provide an important opportunity to determine whether immunoelectronmicroscopy is possible.

2.1 Results: Co-localization of A_1R and $P2Y_1R$ ($P2Y_2$) in transfected HEK293T cells

Confocal imaging for studying the GPCRs using transfected HEK293T cells is the most common method of co-localization of GPCRs. In our laboratory, the co-localization of A_1R and $P2Y_1R$ ($P2Y_2R$) in co-transfected HEK293T cells has been examined by the double immunostaining of HA-A_1R and Myc-$P2Y_1R$ (or HA-A_1R and Myc-$P2Y_2R$) in order to compare localization pattern. Confocal images of co-transfected HEK293T cells double labeled for HA-A_1R (red) and Myc-$P2Y_2R$ (green) are shown in Fig.1. As co-localization occurred upon the plasma membrane, this data supports the heteromeric association of A_1R and $P2Y_2R$. A similar pattern of co-localization for A_1R and $P2Y_2R$ has been demonstrated in rat brain sections as shown in Fig.2.

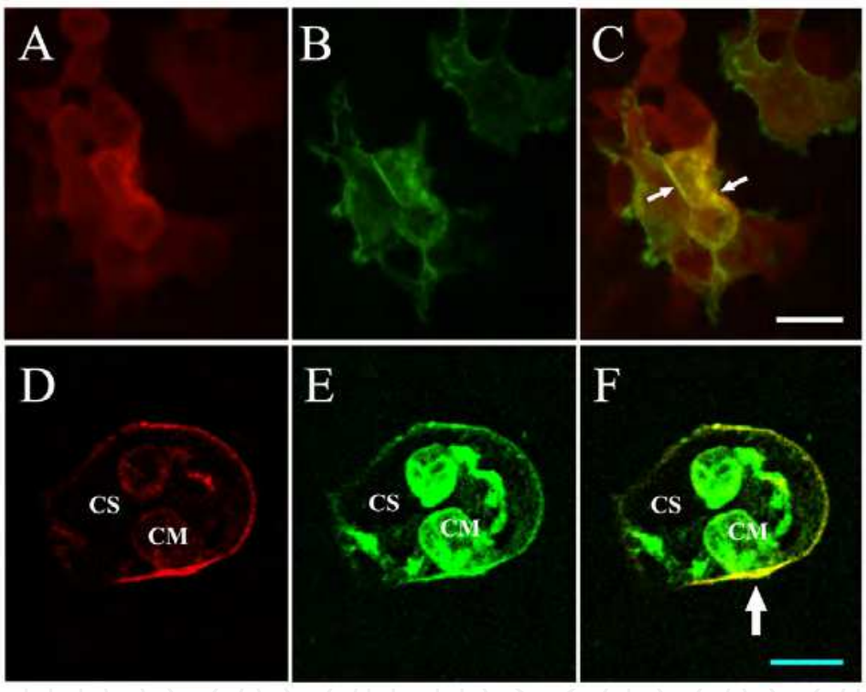

Fig. 1. Co-localization of A_1R and $P2Y_2R$. *A-C.* Confocal images of double immunostained HA-A_1R (A; red), Myc-$P2Y_2R$ (B; green), and their merged images (C; yellow) in co-transfected HEK293T cells. The co-localization of HA-A_1R and Myc-$P2Y_2R$ is evident at the cell surface membrane (C; small arrow). White bar = 50 μm (A-C). Confocal images of double immunofluorescence for HA-A_1R (D; red), Myc-$P2Y_2R$ (E; green), and their merged images (F; yellow) in co-transfected HEK293T cells are also obtained. Co-localizations of A_1R and $P2Y_1R$ (F) was detected upon the cell surface membrane, but was not as evident upon inner cellular membranes (F; arrow). Cyan bar = 10 μm (D-F). Fluorescent images were obtained via confocal laser scanning microscopy (Zeiss LSM410, Carl Zeiss, Oberkochen, Germany) at two levels: 30-μm(A-C), and 15-μm(D-F). At each level, serial images were collected at 1-μm intervals through a total sectional thickness of 40-μm. Serial optical sections were recorded using an air objective lens of (20 X and 40X, numerical aperture; 0.6).

Both receptors were localized predominantly upon the cell surface and cytosolic membranes (Fig. 1. A,B). Merged images showed co-localization mainly in cell membranes (Fig. 1. C.). Our negative controls showed no positive signals in non-transfected HEK293T cells, indicating that the immunoreactivity observed in Fig. 1 was specific to the expressed receptors (data not shown).

2.2 Results: Immunohistochemical studies in rat brain

We examined the expression of A_1R and P2YR in brain using using immunohistochemistry (Yoshioka *et al.*, 2002, Namba *et al.*, 2010). Prominent staining of A_1R and $P2Y_2R$ were observed, particularly in Purkinje cells (Fig. 2A-C). Expression was predominantly restricted to cell bodies and neuronal dendrites. Importantly, co-localization of A_1R and $P2Y_2R$ was observed in cell bodies within the cerebellum, but was detected within the nucleus of Purkinje cells.

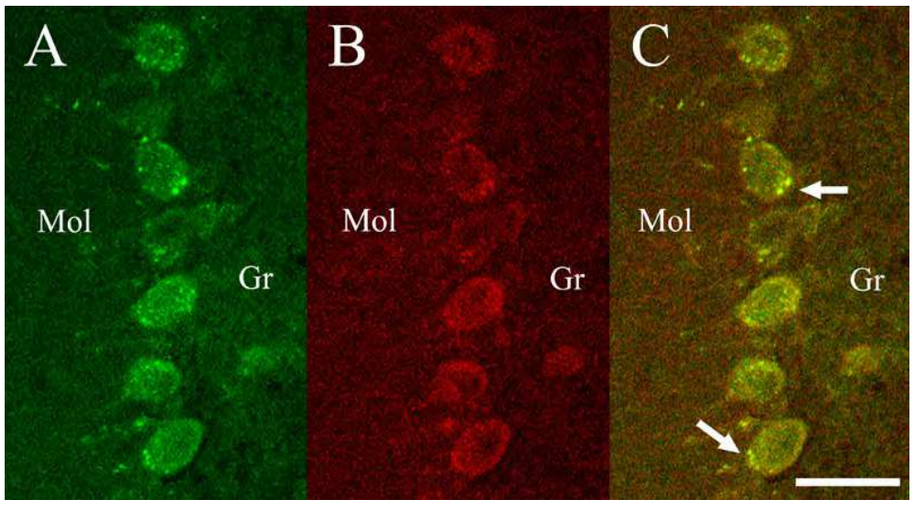

Fig. 2. Confocal images of double immunofluorescence stained A_1R (A; green), $P2Y_2R$ (B; red), and their merged images (C; yellow) in Purkinje cells. Mol: cerebellar molecular layer, Gr: cerebellar granule cell layer. Co-localization of A_1R and $P2Y_2R$ (C; yellow) were detected in the soma of the Purkinje cells (arrows). Bar = 50 μm. Fluorescent images were collected via confocal laser scanning microscopy (Zeiss LSM410, Carl Zeiss) and each 10-μm optical slice consisted of a stack of of 20 sections (0.5-μm thick). Serial optical sections were recorded using an air objective lens of (40X, numerical aperture; 0.6).

3. Pre and post-embedding immunogold electron microscopy of transfected HEK293T cells

The monomeric- or hetero-oligomerization of intrinsic GPCRs cannot be ascertained by immunoelectronmicroscopic examination of brain tissues alone. Data concerning the hetero-oligomerization of GPCRs in brain tissues is typically acquired from three experimental phases. The first phase involves immunoelectronmicroscopic data acquired from pre-embedding methods and gene transfected cells. This provides important information as to whether hetero-expressed GPCRs can oligomerize or not. In other words, this method compares the expression patterns and co-localization of differently-sized immunogold

particles using transfected- or non transfected-cells, yielding data that can determine the occurrence of GPCR hetero-oligomerization when using the same antibodies and immunoreactive conditions. Additionally, a particular advantage of using pre-embedding methods is that native antigenicity is maintained. The second phase is to acquire data from gene transfected cells using post-embedding methods. The reason why immunoelectronmicroscopic observation of tissues is applied with post-embedding methods is because it is difficult for a specific epitope anti-body to penetrate into the cytoplasmic region of tissue cells. Before experimenting on tissues, it is important to confirm patterns of immune-reaction with transfected cells using post-embedding methods in order to form positive controls for specific tissues. The last phase is to acquire data from tissues using post-embedding methods. It is suggested that the hetero-oligomerization of GPCRs in tissues would be very precise as an oligomer of different-sized gold particles in a given case of comparative data from single transfected GPCRs and co-transfected GPCRs.

3.1 Results: Immunogold electron microscopic observations of HA-A_1R and Myc-$P2Y_2$R expressed in transfected cells

In our laboratory, we examined the cellular localization of HA-A_1R/Myc-$P2Y_2$R in co-transfected HEK293T cells using post-embedding methods, anti-HA or anti-Myc IEM (Figs. 3A-D) (Namba *et al*., 2010). Immunogold particles were localized individually or in clusters, indicating that both HA-A_1R and Myc-$P2Y_2$R form monomers and homo-oligomers. Specificities of the gold-labeled anti-HA and anti-Myc antibodies were demonstrated by incubating A_1R-transfected HEK293T cells with a mixture of both antibodies. Data showed that only A_1R-labeled particles were present (Fig. 3A). No significant patterns were detected with either anti-HA and anti-Myc antibodies in mock-transfected HEK293T cells or with only secondary alone (i.e., no primary antibodies) in HA-A_1R-transfected HEK293T cells (data not shown). Also, when Myc-$P2Y_2$R-transfected HEK293T cells were incubated with both anti-HA and anti-Myc antibodies, we detected single particles (monomers) scattered all over the cells (Fig.3B). Another control for hetero-oligomerrization, HA-A_1R-transfected HEK293T cells incubated with both anti-HA and Myc-$P2Y_2$R, we observed all over the cells (Fig. 3D, inner cellular site). In HEK293T cells co-transfected with both HA-A_1R and Myc-$P2Y_2$R, clusters of different-sized particles were observed mainly at the cell surface (Fig. 3C) suggesting the formation of hetero-oligomers.

We would also like to introduce another means of investigating the cellular localization of anti-A_1R/anti-$P2Y_1$R in co-transfected HEK293T cells using post-embedding methods. Using mouse anti-A_1R or rabbit anti- $P2Y_1$R antibodies, hetero-oligomeric gold particles were clearly observed, predominantly at the cell surface (Fig.3C). This pattern concurred with patterns defined using pre-embedding methods and gold-labeled anti-HA and anti-Myc antibodies. The frequency of A_1R and $P2Y_1$R ($P2Y_2$R) hetero-oligomers detected using post- embedding methods was smaller than that detected with pre-embedding methods using fresh specimens. This was likely to be due to polymerization occuring during the embedding process (data not shown). We consider that the native antigenicities of GPCRs in transfected cells may be reduced by polymerization treatment with LR-white, though closely-related patterns of immunoreactivity were obtained in our laboratory across differeing methods (Fig. 4). HA-A_1R transfected HEK293T cells incubated with mouse anti-A_1R using post-embedding methods indicated patterns (Fig. 3A, large particles) identical to

those arising from pre-embedding methods (Data not shown). This data indicates that the immunoreactivety of mouse anti-A_1R antibodies using LR-white post-embedding were effective in HA-A_1R transfected HEK293T cells. Hetero-oligomeric gold particles of the mouse anti-A_1R or rabbit anti-$P2Y_1R$ antibodies were observed at the cell surface (Fig.4B). HA-A_1R-transfected HEK293T cells incubated with either mouse anti-A_1R or rabbit anti-$P2Y_1R$ were also seen scattered all over the cells (Fig. 4C, cellular surface).

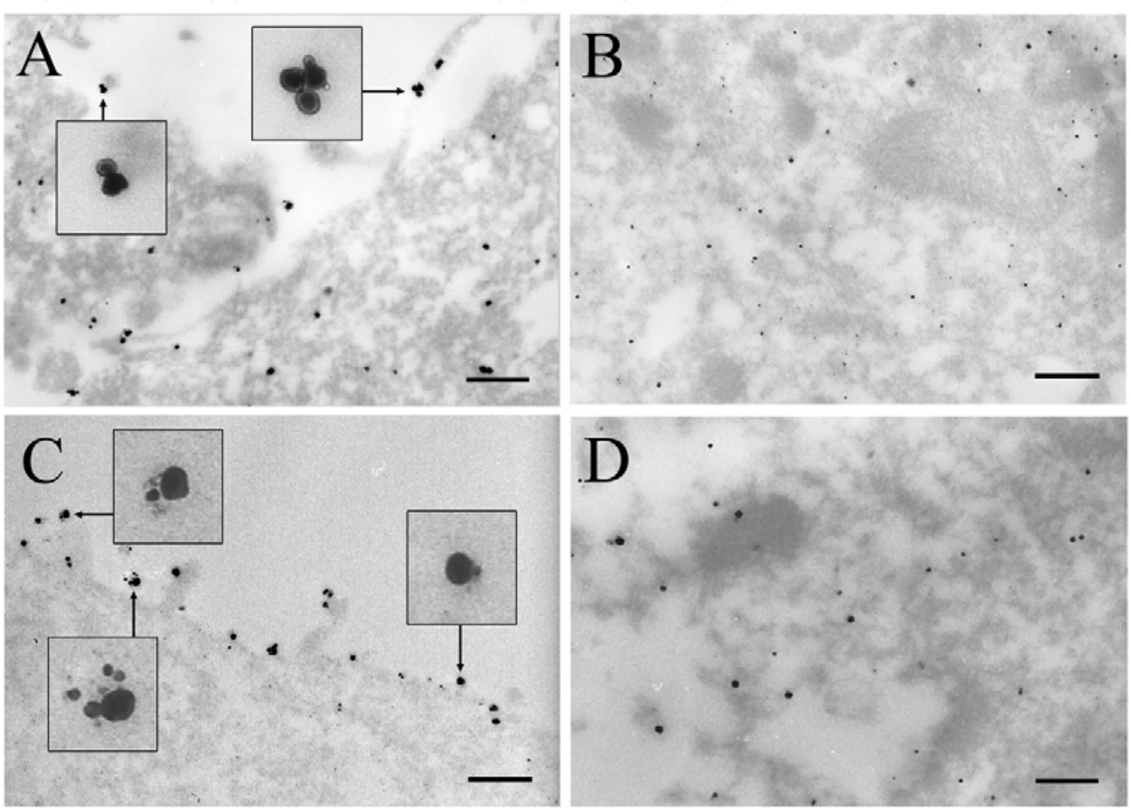

Fig. 3. Immunogold electron microscopy (post-embedding) method to visualise A_1R and $P2Y_2R$ in transfected HEK293T cells using nanogold particles. A: Localization of HA-A_1R (large particles) detected with anti-HA in HA-A_1R-transfected HEK293T cells. B: Localization of Myc-$P2Y_2R$ (small particles) detected with anti-Myc in Myc-$P2Y_2R$-transfected HEK293T cells. C: Anti-HA and anti-Myc immuno-localization of anti-A_1R and Myc-$P2Y_2R$ in co-transfected HEK293T cells. D: HA-A_1R-transfected HEK293T cells incubated with both anti-HA and anti-Myc. Bars represent 100 nm.

4. Post-embedding immunogold electron microscopy of brain tissues

In keeping with observations gained by post-embedding methods for the study of co-transfected HEK293T cells described in Section 3.1. of this chapter, we should highlight that it is also possible to apply immunogold staining using post-embedding methods for the study of brain tissues. Comparing the dose of immunoreactivity from gold particles reflecting hetero-oligomers using co-transfected culture cells and post-embedding methods is essential in acquiring immunogold pattern data from hetero-oligomers *in situ*.

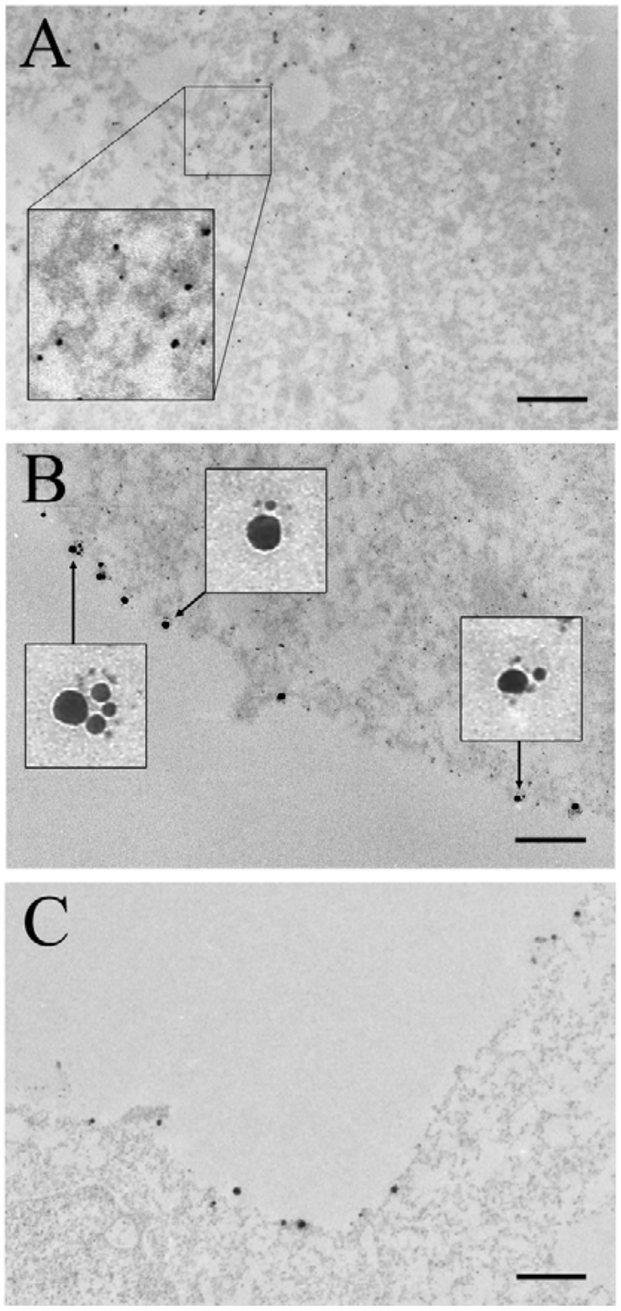

Fig. 4. Immunogold electron microscopy to visualise A_1R and $P2Y_1R$ in transfected HEK293T cells using nanogold particles. A: Localization of HA-A_1R (small particles) detected with mouse anti- A_1R in HA-A_1R -transfected HEK293T cells. B: Mouse anti-A_1R and rabbit anti-$P2Y_1R$ immuno-localization of HA-A_1R and Myc-$P2Y_1R$ in co-transfected HEK293T cells. C: HA-A_1R-transfected HEK293T cells incubated with both mouse anti-A_1R and rabbit anti-$P2Y_1R$. Bars represent 100 nm.

There are two reasons for this. Firstly, the antigenicity for the two receptor antibodies must accurately reflect hetero-oligomers or single expression. Secondly, the immunoreactivity of a

particular antibody could be variable depending upon the methodology utilized, for example whether post- or pre-embedding methods were deployed. Usually, immunoreactive conditions during pre-embedding methods are much better than during post-embedding methods. However, immunoreactions involving inner tissues are technically difficult to perform. In the following section, we introduce how we can image the hetero-oligomerization of A_1R and $P2Y_1R$ in brain tissues using post-embedding immunogold electron microscopy.

4.1 Results: Immunogold electron microscopic observations of A_1R and P2YR expressed in brain tissues

We incubated post-embedded, primary antibody-stained rat brain tissues with two secondary antibodies labeled with gold particles (a 5-nm gold particle-conjugated goat anti-mouse IgG antibody for A_1R, and a 10-nm gold particle-conjugated goat anti-rabbit IgG antibody for $P2Y_1R$). As negative controls, brain tissues were stained with only secondary antibodies conjugated with different sized gold particles; no significant immunoreactivity was observed under the experimental conditions (data not shown). As found with transfected HEK293T cells (3.2-3.4), we observed clusters of different-sized gold particles at cytoplasmic membranes in cell bodies, indicating the presence of heteromeric complexes of endogenous A_1R and $P2Y_1R$ in the rat cerebellum (Fig. 5). Significant immunoreactivity was

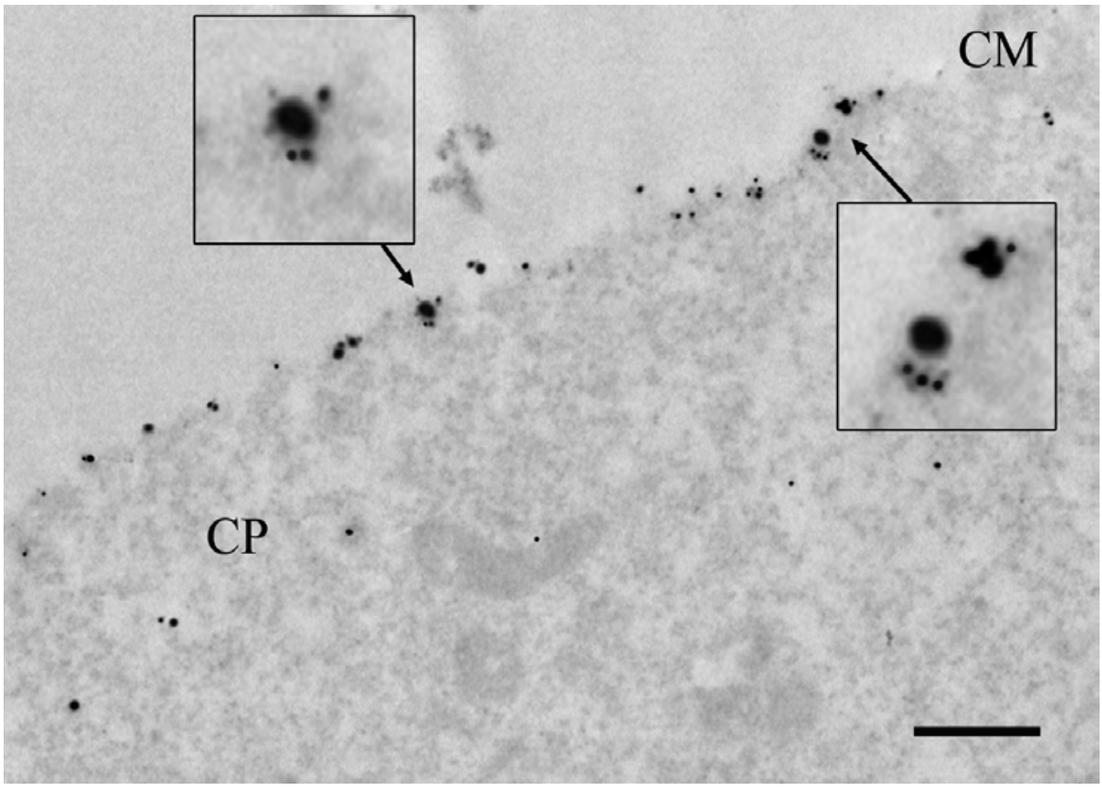

Fig. 5. Immunogold electron microscopy (post-embedding) method for the visualisation of A_1R and $P2Y_1R$ in rat brain using nanogold particles. Localization of A_1R (small particles) and $P2Y_1R$ (large particles) in cell surface of Purkinje cells detected with both anti-A_1R and anti-$P2Y_1R$ Arrows indicate two adjacent receptors on the cell membrane. Bars represent 100 nm. CM, cell membrane; CP, cytoplasm.

detected in the cell surface region (Fig. 3F). In our earlier experiments, oligomerization of A_1R and $P2Y_2R$ in rat brain tissues under the same experimental conditions involved hippocampal pyramidal cells, cerebellum, and pyramidal cells in the forebrain (Namba *et al*., 2010). Hetero- and homo-oligomers of both $A_1R/P2Y_1R$ and $A_1R/P2Y_2R$ were detected in significant numbers at the cell surface in both transfected HEK293T cells and native brains.

5. Data analysis: Comparison of the frequencies of monomers, homo-oligomers, and hetero-oligomers between $P2Y_1R/A_1R$ and $P2Y_2R/A_1R$

Gold-staining was quantified in the following way. Firstly, gene-transfected HEK293T cells exhibiting the highest number of total immuno-reacted gold particles were defined as 100% labeling. Since co-transfected HEK293T cells that displayed unique pharmacology in our previous study (Suzuki *et al*., 2006) exhibited more than 20% hetero-oligomeric gold particles, we used this number as a threshold in the current study. Thus, cells with more than 20% hetero-oligomeric particles were defined as being "significantly stained", and those with 20% or less were defined as "not significantly stained".

The proportions of relative distributions for A_1R and P2YR between cell surfaces and inner cytoplasmic membranes were clearly different (Fig. 6). The tendency for the proportional distribution of A_1R and P2YR at the surface of HEK293T cells concur with data from brain

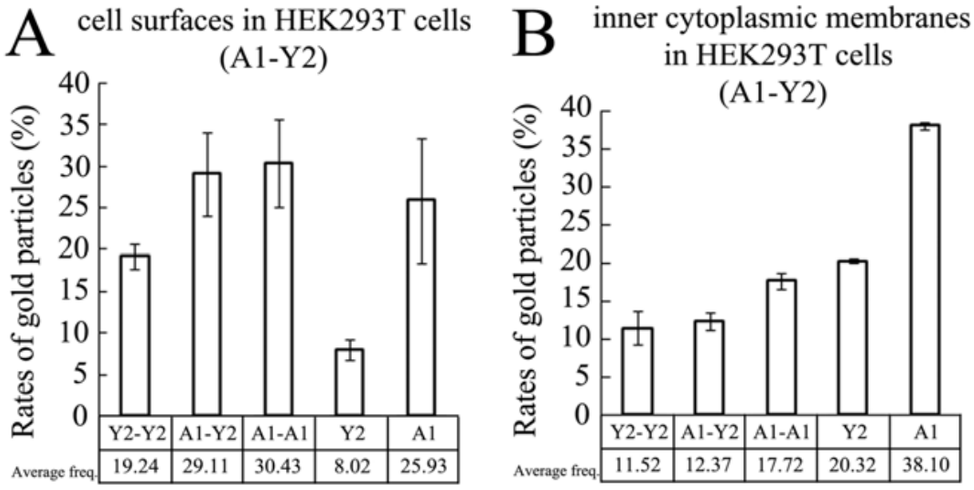

Fig. 6. Comparison of the relative distributions for A_1R- and $P2Y_2R$-conjugated gold particles between the cell surface and inner cytoplasmic membranes.

tissues. Based on this, the numbers of immunogold particles at the surface of each cell type in brain tissues were determined. We defined single particles located independently as monomers (A_1R and P2YR in Fig. 7), complexes composed of clusters of the same-sized gold particles as "homo-oligomers" (A_1R-A_1R or P2YR-P2YR in Fig. 7), and those of different sized gold particles as "hetero-oligomers" (A_1R-P2YR in Fig. 7). Separate calculations were carried out for particles in Purkinje cells (Fig. 7A, C), hippocampal pyramidal neurons (Fig. 7B, D), and cortical neurons (Fig. 7E). To do this, gold particles were counted the number of in three cells for each region. We previously counted immunogold particles in co-transfected HEK293T cells (Namba *et al*., 2010). The total number of immunoreactive gold particles on

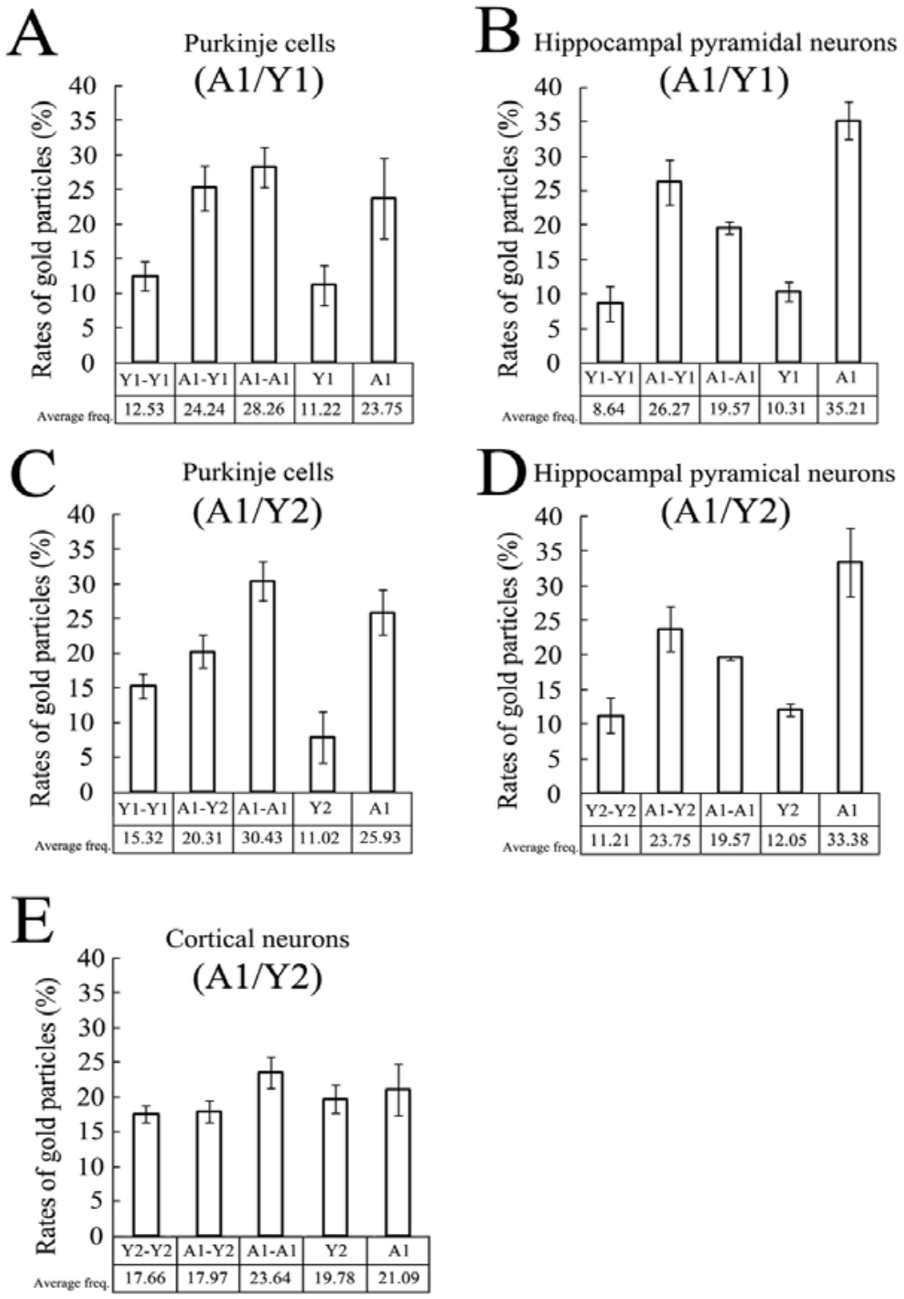

Fig. 7. Bar graphs comparing the relative distributions of A_1R(A1)/$P2Y_1R$(Y1) immunoreactive elements in Purkinje cells (A) Hippocampal pyramidal cells (B), and A_1R(A1)/$P2Y_2R$(Y2) immunoreactive elements in Purkinje cells (C), Hippocampal pyramidal cells (D) and Cortical neurons (E). P2YR-P2YR, A_1R-A_1R and A_1R-P2YR oligomers are indicated by Y1-Y1 (Y2-Y2), A1-A1 and A1-Y1 (A1-Y2), respectively. The total number of immunoreactive gold particles on the cell surface was defined as 100%. Each column represents the average frequency (± SD) from three cells. Data describing the average numbers of gold particles are shown in tables under the graphs. Data represent the mean of three independent experiments.

each cell surface was defined as 100%. From a total of 12 photos from each brain area (i.e., 36 photos) and from transfected cells that were reacted under the same conditions as the brain sections for each immunostaining, we selected three photos of each specimen containing whole cells for comparison.

We then counted gold particles on the surfaces of cells in the cerebellum, hippocampus and cortical neurons, and classified them as monomers, homo-oligomers, or hetero-oligomers. While the homo-oligomerization ratios (A_1R-A_1R/P2YR-P2YR) displayed different patterns between Purkinje cells and hippocampal pyramidal cells, the rates of hetero-oligomerization were particularly prominent in hippocampal pyramidal cells among them. Curiously, the frequency of A_1R or $P2Y_1R$ hetero-oligomerization was slightly higher than that of A_1R or $P2Y_2R$ in both tissues (Fig. 7A, B). This indicated that the hetero-oligomerization of A_1R or $P2Y_1R$ are the dominant form in both Purkinje cells and hippocampal pyramidal cells.

6. Discussion

Previous reports describe electron microscopic studies of plasma membranes for homo-oligomeric B_1 Bradykinin receptor complexes (Kang *et al*., 2005), heteromeric-oligomerization of $GABA_B$ R1 and R2 receptors (Charara *et al*., 2004), and the localization of A_1R with caveolin-3 in rat ventricular cardiomyocytes (Lasley *et al*., 2000). An immunological study suggested that A_1R forms oligomers the cortex of the pig brain (Ciruela *et al*., 1995), and a FRET study demonstrated the oligomerization of $P2Y_2R$ in transfected HEK293 cells (Kotevic *et al*., 2005). The hetero-oligomerization of A_1R-$P2Y_1R$ on postsynaptic neurons was also analyzed by IEM (Tonazzini *et al*., 2007). The present study provides the first detailed evidence of an interaction between endogenous A_1R and $P2Y_2R$ in brains using IEM.

The homo- oligomerization of A_1R and its structural profile were previously analyzed in our laboratory by computational prediction, co-immunoprecipitation, and BRET analysis with differently tagged A_1Rs (Suzuki *et al*., 2009); homo-oligomers and monomers were easily distinguished by IEM. This particular study confirmed the existence of homo-oligomers (A_1R-A_1R and $P2Y_2R$-$P2Y_2R$) using IEM. Interestingly, the percentage of A_1R homo-oligomers was higher than that of $P2Y_2R$ in both rat brain and transfected HEK293T cells (Namba *et al*., 2010). By contrast, the ratio of heteromeric gold-particle clusters were different in the cortex, hippocampus, and cerebellum. Importantly, both homo-oligomeric and hetero-oligomeric gold-particles were reduced in number at inner cytoplasmic membranes than at the cell surface (data not shown). In general, most GPCRs oligomers have been observed at the cell surface (Minneman, 2007; Bulenger *et al*., 2005).

While the frequencies of A_1R and $P2Y_1R$ homo-oligomers and monomers were similar in the cerebellum (Fig. 5) and in transfected HEK293T cells (Fig. 4B), the ratio of the different receptor oligomers occurred in different patterns in each of the three brain areas (Fig. 7). Total numbers of hetero-oligomers observed on the cell surface and in the cytoplasm were clearly different (Fig. 6A, B) and may reflect the process of receptor maturation and association of the A_1R-P2YR complex. However, hetero-oligomers were unmistakably detected at the cell surface by IEM (Fig. 3C, Fig. 4B).

As a signaling pathway, $P2Y_1R$ and $P2Y_2R$ display different ligand specificities. As ligands, ATP and UTP fully activate $P2Y_2R$. However, UTP is not an agonist for $P2Y_1R$. In addition,

ADP is a strong agonist for $P2Y_1R$ but not $P2Y_2R$ (Abbracchio *et al*., 2006). Many previous studies suggest that A_1R-$P2Y_1R$ and A_1R-$P2Y_2R$ hetero-oligomers exhibit general pharmacological profiles, possibly because of differences in the conformational changes induced by oligomerization (Nakata *et al*., 2010). The hetero-oligomerization of A_1R-$P2Y_1R$ inhibits adenylyl cyclase activity via the $G_{i/o}$ protein linked effector. The hetero-oligomerization of A_1R-$P2Y_2R$ resulted in an increase in intracellular Ca^{2+} levels induced by $P2Y_2R$ activation of $G_{q/11}$ which was synergistically enhanced by the simultaneous addition of an A_1R agonist in the co-expressing cells (Suzuki *et al*., 2006). Differences in the amounts of hetero-oligomerization between A_1R-$P2Y_1R$ and A_1R-$P2Y_2R$ were observed (Fig. 7). Assuming that the number of hetero-oligomers formed is functionally dominant, the dominancy of the signaling via A_1R-$P2Y_1R$ may be generated by competitive antagonism in pharmacology between $P2Y_1R$ and $P2Y_2R$ in order to oligomerize with A_1R. This hypothesis, however, requires further investigation.

In our previous study, the hippocampal hetero-oligomerization of A_1R and $P2Y_2R$ was far more pronounced than in other regions of the brain (Namba *et al*., 2010). Another research group suggested that the hetero-oligomerization, or cross-talk between A_1R and $P2Y_1R$ is involved in regulation of glutamate release in the hippocampus (Tonazzini *et al*., 2007). The relative distributions of immunoreactivity for $GABA_B$ R2 and $GABA_B$ R1 were also different in the basal ganglia and globus pallidus/substantia nigra, which suggests the possible co-existence and hetero-oligomerization of the two types of receptors at various pre-/postsynaptic sites (Charara *et al*., 2004). From the present study, it can be speculated that the $A_1R/P2Y_2R$ hetero-oligomer might be responsible for down regulation, via hippocampal Ca^{2+} secretion, of synaptic functions (Safiulina *et al*., 2006). Furthermore, the abundant formation of $A_1R/P2Y_1R$ or $A_1R/P2Y_2R$ hetero-oligomers in the cerebellum revealed in this present study supports the idea that the unique signal transduction generated by hetero-oligomerization, including the enhancement of Ca^{2+} signaling via $G_{q/11}$, observed in transfected cells, also occurs in the cerebellum.

There are many families of GPCRs expressed in whole brain, most of which remain a mystery. However, it is clear that GPCR hetero-oligomerization is common in the brain and exhibits unique pharmacology in this region, thus implying that associated signal transduction pathways can be anticipated in this region. The methodology described here using immunogolod particles is one of the most influential techniques available to elucidate the ingenious mechanism underlying GPCR hetero-oligomerization.

7. Summary

In summary, IEM provided direct evidence for the existence of homo- and hetero-oligomers of A_1R and $P2Y_2R$, not only in co-transfected cultured cells, but also *in situ* on the surface of neurons in various brain regions. The molecular mechanisms responsible for the control of A_1R and P2YR monomer/homo-oligomer/hetero-oligomer ratios remain to be elucidated. Future investigation of GPCR oligomer formation is indispensable for revealing the elaborate mechanisms of cellular function.

The importance of these novel experimental procedures using IEM is to provide information concerning crosstalk between small molecules with high angle views of whole cells, although these methods do require a high level of technical skill. The development of ingenious histochemical and immunoelectronmicroscopic methods has made it possible to

visuallize crosstalk and provide specific insight into the nature of hetero-oligomers of not only GPCRs, but also various proteins expressed by cells.

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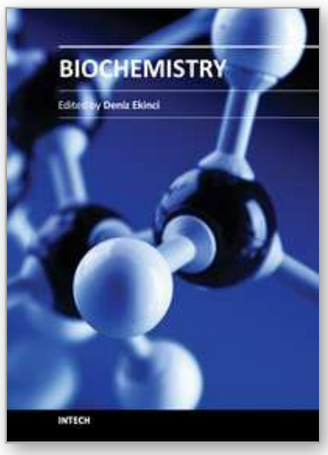

Biochemistry

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Over the recent years, biochemistry has become responsible for explaining living processes such that many scientists in the life sciences from agronomy to medicine are engaged in biochemical research. This book contains an overview focusing on the research area of proteins, enzymes, cellular mechanisms and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in biochemistry. Particular emphasis is devoted to both theoretical and experimental aspect of modern biochemistry. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in biochemistry, molecular biology and associated areas. The book is written by international scientists with expertise in protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the book will enhance the knowledge of scientists in the complexities of some biochemical approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of biochemistry.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
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