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The $P2Y_4$ receptor forms homo-oligomeric complexes in several CNS and PNS neuronal cells

Nadia D'Ambrosi · Monia Iafrate · Fabrizio Vacca · Susanna Amadio · Alessandro Tozzi · Nicola B. Mercuri · Cinzia Volonté

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Abstract It is well established that several cell surface receptors interact with each other to form dimers and oligomers, which are essential for their activation. Since little is known about the quaternary structure of P2Y receptors, in the present work, we investigated the expression of the G-protein-coupled $P2Y_4$ subunit as monomeric or higher-order complex protein. We examined both endogenously expressed $P2Y_4$ subtype with the aid of specific anti-$P2Y_4$ antiserum, and heterologously transfected $P2Y_4$-tagged receptors with the use of antitag antibodies. In both cases, we found the $P2Y_4$ receptor displaying molecular masses corresponding to monomeric, dimeric and oligomeric structures. Experiments performed in the absence of reducing agents demonstrated that there is a strict correlation among the multiple protein bands and that the multimeric forms are at least partially assembled by disulphide bonds. The direct demonstration of $P2Y_4$ homodimerisation comes instead from co–transfection and differential co–immunoprecipitation experiments, with the use of differently tagged $P2Y_4$ receptors and antitag antibodies. The structural propensity of the $P2Y_4$ protein to form homo-oligomers may open the possibility of a novel regulatory mechanism of physiopathological functions for this and additional P2Y receptors.

Key words G-protein-coupled receptors · PC12 cells · purinergic receptor · receptor dimerisation · SH-SY5Y cells

Abbreviations

CGN	Cerebellar granule neurons
DTT	Dithiothreitol
FCS	Foetal calf serum
GFP	Green fluorescent protein
GPCRs	G-protein-coupled receptors
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulphate

Introduction

Molecular cloning and heterologous receptor expression studies have led to the identification and characterisation of eight different P2Y receptor subtypes ($P2Y_{1,2,4,6,11–14}$) [1]. $P2Y_{1,2,4,6,11}$ receptors display 25–52% amino acid identity and couple, via heterotrimeric G proteins of the Gq family, to activation of phospholipase C, generation of inositol phosphates, and mobilisation of intracellular Ca^{2+} stores [2, 3]. Only the $P2Y_{11}$ receptor also couples to Gs proteins to activate adenylyl cyclase and promote cyclic AMP accumulation [4–6]. The recently identified $P2Y_{12–14}$ receptors have high sequence identity with each other (40–48%), but share relatively little identity (22–25%) with the other P2Y receptors. Moreover, differently from $P2Y_{1,2,4,6,11}$ receptors, they are associated with Gi proteins, to inhibit adenylyl cyclase [7, 8]. P2Y receptors can alternatively be classified

N. D'Ambrosi · M. Iafrate · F. Vacca · S. Amadio · A. Tozzi · N. B. Mercuri · C. Volonté
Fondazione Santa Lucia,
Rome, Italy

N. D'Ambrosi · N. B. Mercuri
Department of Neuroscience, University of Rome Tor Vergata,
Rome, Italy

C. Volonté
C.N.R. Institute of Neurobiology and Molecular Medicine,
Rome, Italy

C. Volonté (✉)
Fondazione Santa Lucia at CERC,
Via del Fosso di Fiorano,
64-00143 Rome, Italy
e-mail: c.volonte@inmm.cnr.it

into purinergic and pyrimidinergic subclasses. In particular, $P2Y_{1,11,12,13,14}$ receptors are activated by adenine nucleotides [1]; $P2Y_2$ and rat $P2Y_4$ subunits display no selectivity between ATP and UTP [9]; human $P2Y_4$ ($hP2Y_4$) and $P2Y_6$ subtypes are mainly pyrimidinergic [10], but with ATP behaving as a potent competitive antagonist at the $hP2Y_4$ subunit [11].

One of the most broadly expressed P2Y receptor in various brain regions is $P2Y_4$. Highest mRNA levels are present in the cerebellum, hypothalamus, medial frontal gyrus, parahippocampal gyrus, striatum and thalamus; lowest expression levels were detected in the amygdala and spinal cord [12]. Moreover, $P2Y_4$ mRNA was found in the dorsal root, nodose and trigeminal ganglion [13]. In situ hybridisation studies confirmed a similar wide distribution, with highest incidence in the pineal gland and ventricular system [14]. At the protein level, the presence of the $P2Y_4$ receptor was demonstrated by immunohistochemistry in sensory ganglia [13], the brainstem and ventral white matter of the spinal cord during rat brain development [15].

Although according to amino acid sequence the predicted molecular mass of the $P2Y_4$ protein is 41 kDa [14], Western blot studies identified a protein corresponding to 88 kDa in rat cerebellar granule neurons [16, 17]. This same high molecular weight was demonstrated in glutamatergic neurons from rat hippocampus, in astrocytoma 1321N1 cells stably transfected with $P2Y_4$ receptor [18] and in NGF-differentiated PC12 cells [19]. In rat oligodendrocyte precursors [20] and rat cortical astrocytes [21], the 88 kDa protein was associated with a lower molecular mass band, of about 45 kDa, while in gerbil brain, the $P2Y_4$ receptor displayed three bands (respectively, 75, 55 and 36 kDa) [22]. While classical studies postulated that G-protein-coupled receptors (GPCRs) exist only as monomers, many recent findings instead demonstrated that many GPCRs form dimers and oligomers [23]. For example, it was recently demonstrated that $P2Y_1$ receptor forms heterocomplexes with A1 proteins [24–26] and that $P2Y_4$ coimmunoprecipitates with NMDA receptors [17].

Since only little is known specifically on the quaternary structure of P2Y receptors, the purpose of the present study was to analyse the potential expression of the $P2Y_4$ subunit in monomeric and higher-order complex proteins. The attainment of this knowledge could be of great importance in understanding the regulation and function not only of the $P2Y_4$ subtype but also of additional P2Y receptors.

Materials and methods

Cell line cultures

Rat phaeochromocytoma PC12nnr5 cells were cultured on collagen-coated dishes in RPMI 1640 (Gibco BRL, MI, Italy) medium supplemented with 10% heat-inactivated horse serum and 5% foetal calf serum (FCS) (Gibco BRL). Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma-Aldrich, MI, Italy) supplemented with 10% FCS. All culture media were supplemented with glutamine (2 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml), and all cell lines were grown at 37 °C in 5% CO_2.

Dissociated primary cell cultures

Cerebellar granule neurons (CGN) from Wistar 8-day-old rat cerebellum were prepared as described [27] and seeded on poly-L-lysine-coated dishes, in Eagle's basal medium (BME) (Gibco BRL), with 25 mM KCl, 2 mM glutamine, 0.1 mg/ml gentamycin, and 10% heat inactivated FCS. At 1 day in vitro, cultures were supplemented with 10 μM cytosine arabinoside and kept for 9 days, without replacing the culture medium.

Construction of epitope-tagged human $P2Y_4$ receptor and transient transfection

The plasmid containing the cDNA encoding for human $P2Y_4$ was a kind gift from Prof. T.K. Harden. cDNA was digested with *EcoRI* and *XhoI* restriction enzymes and ligated into a pCMV expression vector that incorporated a FLAG epitope tag (DYKDDDDK) at the 5′ terminus. Moreover, by the use of polymerase chain reaction (PCR) with Platinum *Pfx* polymerase (Invitrogen, Paisley, UK), human $P2Y_4$ cDNA was amplified with oligonucleotides containing *EcoRI* and *XhoI* restriction sequences for the subcloning into a pCS2 plasmid containing myc epitope tags (EQKLISEEDL) at the 5′ terminus. All constructs were verified by sequencing analysis. SH-SY5Y cells in 35-mm plates were transiently transfected with epitope-tagged P2Y receptor cDNAs individually or in combination. Cells were incubated with 4 μg of total DNA and 4 μl of Lipofectamine™ 2000 (Invitrogen) in 2 ml of serum-free medium (Opti-MEM, Invitrogen) for 24h.

Cell protein extraction

In order to isolate total protein extracts, cells were harvested with ice-cold RIPA buffer [phosphate-buffered saline (PBS), 1% Nonidet P-40-NP-40-, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS)], whereas for immunoprecipitation experiments, cells were lysed in buffer G (1% Triton X-100, 10% glycerol, 150 mM NaCl, 1,5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA). Both buffers were added with 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 μg/ml leupeptin. Cellular lysates were kept for 30 min on ice and then centrifuged for 10 min at 14,000 × g at 4°C.

Supernatants were collected and assayed for protein quantification by the Bradford method [28].

Immunoprecipitation

Purified cell lysates (100 μg) were combined with 15 μl (packed gel) of either anti-c-Myc or anti-FLAG M2 affinity agarose (Sigma-Aldrich) and kept for 2 h at 4 °C on a rotator. The immunoadsorbents were recovered by centrifugation for 2 min at 3,000 × g and washed three times by resuspension centrifugation for 2 min at 3,000 × g in buffer G. The samples were eluted in 30 μl of sample buffer used for SDS-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE and Western blotting analysis

Analysis of protein components was performed on 10% polyacrylamide gels, as described (Laemmli, 1970), loading the same amount of protein for each sample. Gels were transferred onto nitrocellulose membranes (Amersham Biosciences, Cologno Monzese, Italy), and blots were probed overnight at 4 °C, with anti-$P2Y_4$ antiserum (Alomone Labs, Jerusalem, Israel), (1:300), which specificity was directly tested by immunoreactions in the presence of the neutralising peptide (ratio 1:1 between peptide and antiserum) or anti c-myc (9E10) antibody (Chemicon International Inc., Temecula, CA, USA), (1:200). Subsequently, blots were incubated for 1 h with horseradish peroxidase-coupled anti-rabbit (1:5,000) or anti-mouse (1:2,500) antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA), and visualised using electrogenerated chemiluminescence (ECL) (Amersham Biosciences). Image analysis and quantifications were performed by Kodak Image Station (KDS IS440CF 1.1) with 1-dimensional (1D) image analysis software.

Immunofluorescence analysis

SH-SY5Y cells were fixed in 4% paraformaldehyde for 15 min, washed three times with PBS, permeabilised with 0.2% Triton X-100 for 5 min, rinsed with PBS and incubated with PBS containing 0.1% bovine serum albumin (BSA) for 30 min. Anti-c-Myc antibody was added at a dilution of 1:200 in PBS/BSA for 1 h. Cells were washed and stained with Cy2-conjugated donkey anti-mouse IgG (1:100, Jackson Immunoresearch, West Grove, PA, USA) for 1 h, rinsed again and coverslipped with gel/mount™ anti–fading medium (Biomeda, Foster City, CA, USA). Immunofluorescence was analysed by means of a confocal laser scanning microscope (CLSM) (LSM 510, Carl Zeiss, Arese, MI, Italy) equipped with an argon laser emitting at 488 nm, a helium/neon laser emitting at 543 nm and a helium/neon laser emitting at 633 nm.

Intracellular [Ca^{2+}] measurements

Changes in intracellular calcium levels were investigated by a microfluorometry imaging technique. Briefly, cells were grown on glass coverslips to ~80% confluence and transfected with pGreen Lantern™-1 (Gibco BRL) together with wild-type $P2Y_4$ or tagged $P2Y_4$ (1:8 molar ratio). After 24 h, cells were loaded with 1 mM Fura-2/AM for 30 min, washed and placed in a recording chamber mounted on the stage of an upright microscope (Axiscop2 FS, Carl Zeiss, AG, Germany) equipped for infrared video microscopy and microfluorimetry (Till Photonics GmbH, Gräfelfing, Germany). The coverslip was continuously superfused at 2.5 ml/min with artificial cerebrospinal fluid (NaCl 126 mM, KCl 2.5 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 2.4 mM, glucose 10 mM, $NaHCO_3$ 19 mM, pH 7.4, equilibrated with a mixture of O2 95% / CO2 5%). UTP (100 μM) was delivered to the cells by bath applications in the extracellular medium through a three-way tap syringe for 2 min. An area of interest containing green fluorescent protein (GFP) positive cells was chosen and excited for 10 ms alternatively at 340 and 380 nm wavelength for Fura-2 stimulation at 0.5 Hz. The emitted fluorescence was acquired and digitalised by a charge-coupled device (CCD) camera (Photonic Science, Millham, UK). For off line analysis of the images, four regions of interest (ROI) that included the fluorescence areas coming from the cell body of selected GFP-positive cells were measured. Each image was background subtracted, and a ratio of F340/F380 was calculated.

Statistical analysis

Statistical analysis was carried out using a two-way ANOVA, followed by a post-hoc (Newman–Keuls) test for multiple comparisons. A value of $p < 0.05$ was considered significant: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

Different neuronal cell lines display two protein bands for $P2Y_4$ receptor

One of the earliest evidence that a receptor might form dimers or multimers is the presence of multiple bands on Western blot analysis. Therefore, we performed Western blots of total protein extracted from rat phaeochromocytoma PC12 cell variant nnr5 and human neuroblastoma SH-SY5Y cell line, probed with anti-$P2Y_4$ antiserum (Figure 1). In both cell lines, we found the presence of a low-molecular-mass band matching the predicted monomeric $P2Y_4$ receptor (41 kDa) and a higher-order band with a

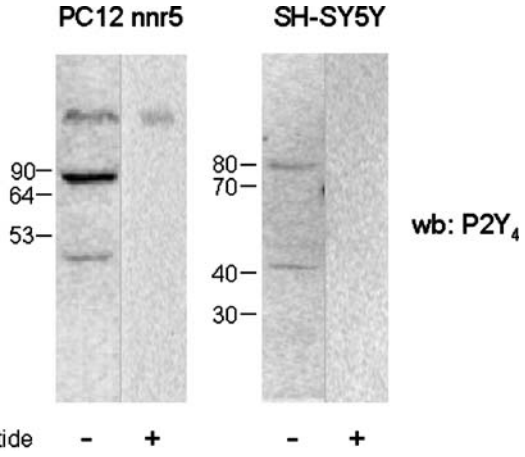

Figure 1 Western blotting detection of $P2Y_4$ receptor in PC12nnr5 and SH-SY5Y cells. Total protein isolated from PC12nnr5 and SH-SY5Y were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gel and transferred onto nitrocellulose membrane. Filters were probed with anti-$P2Y_4$ antiserum in the absence (−) or presence (+) of the antigen peptide. Protein bands were detected with secondary anti-rabbit antiserum coupled to horseradish peroxidase by electrogenerated chemiluminescence (ECL)

molecular mass of about 80 kDa. The occurrence of the two bands is obtained by standard reducing [50 mM dithiothreitol (DTT) and sample boiling] and denaturing (SDS-PAGE) conditions. Moreover, both bands raised specific signals, as evidenced by the lack of immunoreaction after preadsorption of the $P2Y_4$ antiserum with the antigen peptide. These data support the hypothesis that both rat and human $P2Y_4$ receptors form stable dimers.

Tagged $P2Y_4$ receptor is functional and displays multiple protein bands

In order to confirm the existence of a high-molecular-mass complex of the $P2Y_4$ receptor, bypassing the employment of the $P2Y_4$ polyclonal antiserum (even though affinity purified on immobilised antigen), we constructed tagged $P2Y_4$ receptors to be expressed in SH-SY5Y cells and identified with the aid of tag-specific antibodies. Therefore, after transient transfection of SH-SY5Y cells with Myc-$P2Y_4$ cDNA, we first proved by immunofluorescence that the receptor was indeed expressed (we found expression in 25–30% of total cells, data not shown) and displayed a distinguishable cytoplasmic and membrane localisation (Figure 2a). We next evaluated whether the epitope-tagged subunits retained their functional integrity by intracellular Ca^{2+} mobilisation measurements (Figure 2b–e). The addition of 100 μM UTP gave a slight Ca^{2+} transient in control SH-SY5Y cells (probably due to naive $P2Y_4$ and $P2Y_6$ receptors) (Figure 2b) while it determined a marked increase in intracellular Ca^{2+} in cells expressing tagged $P2Y_4$ receptors (Figure 2d, e), with kinetic properties similar to their $P2Y_4$-transfected wild-type parent (Figure 2c, e). In addition, the immunoprecipitation of transfected Myc-$P2Y_4$ with anti-c-Myc antiserum (developed in rabbit) and further detection with anti-c-Myc (9E10) antibody (developed in mouse) displayed three signals, which were instead completely absent in the mock-transfected sample (Figure 3). As for endogenously expressed $P2Y_4$, we found a low-molecular-mass signal at approximately 49 kDa, corresponding to the monomeric Myc-$P2Y_4$ receptor and a higher molecular band (about 100 kDa), which again resembles a potential dimeric form of the $P2Y_4$ subunit. This band seems peculiar for the $P2Y_4$ receptor since transfected Myc-$P2Y_1$, -$P2Y_2$, -$P2Y_6$ and -$P2Y_{11}$ immunoprecipitated by the same anti-c-Myc antibody used for Myc-$P2Y_4$ did not show any comparable denaturing- and reducing-resistant signal (data not shown). Moreover, in the upper part of the gel, we also found a diffuse intense signal that we predict might correspond to oligomeric forms of the $P2Y_4$ receptor.

These findings show that the ability of endogenous $P2Y_4$ to form high-order complexes is a property not only shown

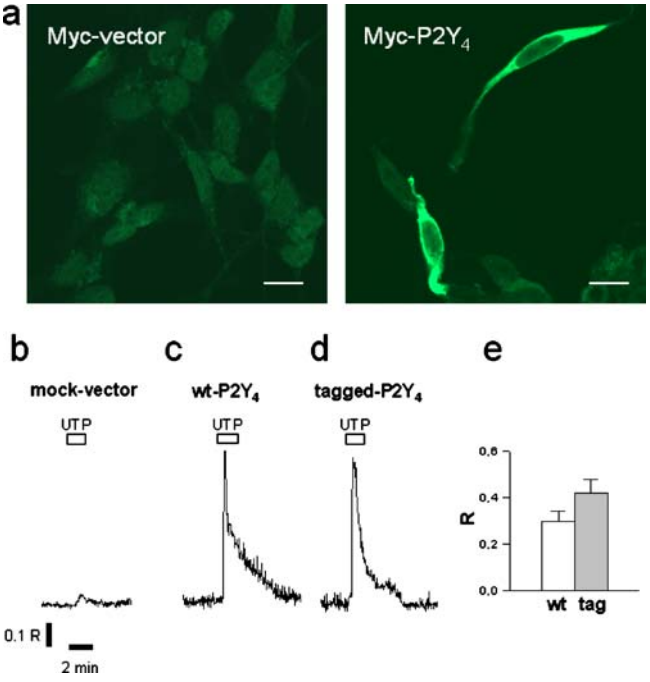

Figure 2 Expression and function of tagged $P2Y_4$ receptors. SH-SY5Y cells transiently transfected with Myc-vector or Myc-$P2Y_4$ were fixed, stained with anti-Myc (9E10) antibody and visualised by Cy2-conjugated anti-mouse antibody by means of a confocal laser-scanning microscope. *Scale bar*, 10 μm (a). Cells transfected for 24 h with mock plasmid, wild-type $P2Y_4$ or tagged $P2Y_4$ in combination with green fluorescent protein (GFP)-expressing plasmid were loaded with Fura-2/AM and stimulated with 100 μM UTP for 2 min. Single traces show the time-course of ratio values (R, F340/F380) and are representative of intracellular Ca^{2+} mobilisation of each condition reported (b–d). The histogram (e) represents the mean amplitudes ± standard error of mean (SEM) of the peak of background-subtracted ratio values in respect to baseline of 18 GFP-positive wild-type transfected cells (*wt*) and 28 GFP-positive tag-transfected cells (*tag*)

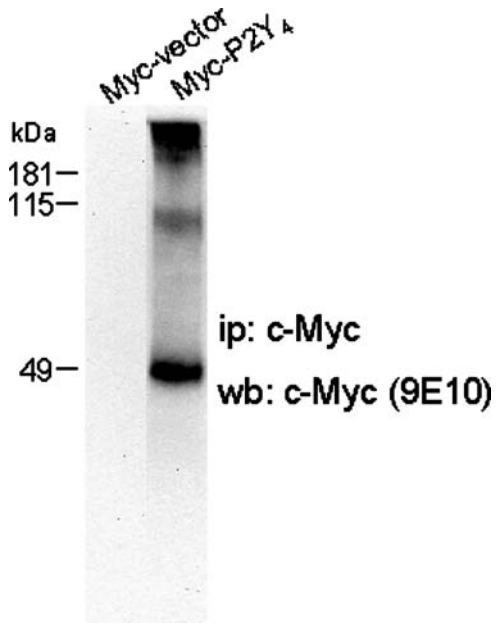

Figure 3 Western blotting pattern of Myc-$P2Y_4$ receptor. SH-SY5Y cells transiently transfected with Myc-vector or Myc-$P2Y_4$ were lysed and immunoprecipitated with anti-c-Myc-conjugated agarose. Immunoadsorbents were run on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), blotted and probed with anti-Myc (9E10) antibody. Protein bands were detected with secondary anti-mouse antiserum by electrogenerated chemiluminescence (ECL)

in different cell types in different species but also shared with recombinant Myc-$P2Y_4$.

Reducing conditions alter the monomeric and multimeric ratio of $P2Y_4$ receptor

We next analysed the effects of reducing agents on the multiple expression of $P2Y_4$ protein (Figure 4). In the presence of 50 mM DTT and 5% β-mercaptoethanol (β-ME), 80% of the total $P2Y_4$-positive signal is given in CGN by the 40 kDa monomeric band while the remaining 20% is constituted by the 80 kDa dimeric signal. This ratio is then inverted when protein separation is performed in the absence of reducing agents: the monomeric band is reduced to 25%, and the higher molecular mass band is increased to 70% of total protein (Figure 4a). A similar situation occurred for the transfected Myc-$P2Y_4$ receptor. Under reducing conditions, the oligomer represented 35% of the total $P2Y_4$ (instead of 55% under nonreducing conditions), the dimer was reduced to 5% (from 20%) and the monomer reached 65% (instead of 30%) (Figure 4b).

Myc-$P2Y_4$ and FLAG-$P2Y_4$ coexpresssed in SH-SY5Y cells associate to form homo-oligomeric complexes

To provide further direct evidence that $P2Y_4$ subunits can form homo-oligomeric structures and to test their direct interaction and coassembly, both the Myc- and FLAG-tagged $P2Y_4$ cDNAs were simultaneously cotransfected in SH-SY5Y cells. As shown in Figure 5, only under this condition, the immunoprecipitate of the FLAG-$P2Y_4$ protein contained its cognate Myc-$P2Y_4$ subunit, as detected by Western blot using the anti-Myc (9E10) antibody. The signal was absent after each single transfection, thus eliminating any possibility of cross-reaction of the antibodies. Moreover, the absence of signal after in vitro mixing of both the Myc- and FLAG-$P2Y_4$ extracts, also ruled out the possibility that the interaction was caused by nonspecific receptor aggregation occurring in vitro during cell lysis and detergent protein extraction.

Discussion

Extracellular nucleotides elicit very heterogeneous physiological effects, by activating not only ionotropic P2X

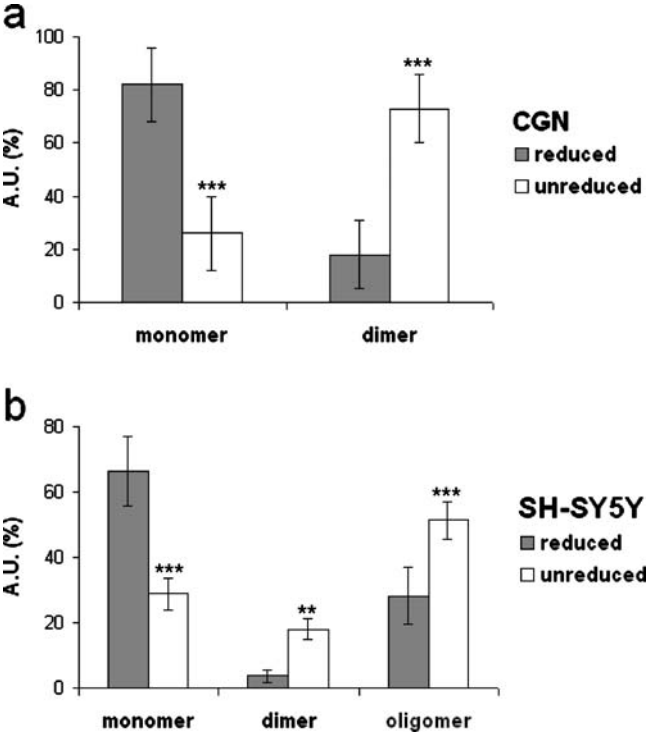

Figure 4 $P2Y_4$ protein expression under reducing and nonreducing conditions. Total protein from cerebellar granule neurons (CGN) (a), and Myc-precipitated proteins recovered from Myc-$P2Y_4$- transfected SH-SY5Y cells (b) were run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the absence or presence of 5% β-mercaptoethanol (β-ME) and 50 mM dithiothreitol (DTT). After immunoblotting with anti-$P2Y_4$ or anti-c-Myc (9E10) antibodies, band intensity [expressed as arbitrary units (A.U.)] was analysed by Kodak 1.D Image Analysis software and reported as percent of total protein expression. Data represent means ± standard error of mean (S.E.M) from three (a) and five (b) independent experiments. Statistical analysis was performed using a two-way ANOVA followed by a post-hoc (Newman–Keuls) test for multiple comparisons (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

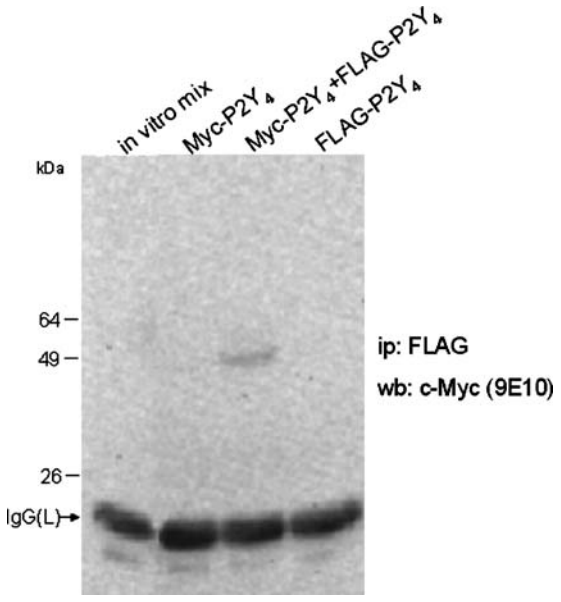

Figure 5 Homo-dimerisation of tagged-$P2Y_4$ receptor. SH-SY5Y cell were transiently transfected with Myc-$P2Y_4$, FLAG-$P2Y_4$ or a combination of both tagged receptors. Solubilised proteins were immunoprecipitated with the anti-FLAG resin and detected by Western blot with the anti-Myc antibody. Protein bands were detected with secondary anti-mouse antiserum by electrogenerated chemiluminescence. The *first lane* (in vitro mix) derives from cells expressing individual tagged receptors that were mixed during lysis and then immunoprecipitated and detected at the above-mentioned conditions

receptors, but also G-protein-coupled P2Y metabotropic receptors, which are both widely distributed in the central nervous system (CNS) and peripheral nervous system (PNS), in neuronal and glial cells. P2Y receptors are generally present as 308–379 amino acid proteins, with a mass ranging from 41 to 53 kDa after glycosylation. The architecture of P2Y was studied by structural comparison based on sequence analysis, mutagenesis and homology modelling and established that they possess tertiary structures with seven transmembrane domains, extracellular N-terminus and intracellular C-terminus. Very little is known, of the possible quaternary structure of P2Y receptors. In this context, our work greatly improves the current knowledge about metabotropic receptors, demonstrating that at least the $P2Y_4$ subunit can form higher-order complexes. These multimers appear stable, being to some extent resistant to denaturing and reducing conditions, thus indicating that they derive, at least in part, from covalent disulphide bonds occurring between the subunits. Moreover, both rat and human endogenous $P2Y_4$ receptors, originating either from cell lines or primary neurons from both the PNS and CNS, appear as stable dimers, and this occurs also for the heterologous $P2Y_4$ receptor transiently transfected in the neuroblastoma SH-SY5Y cell line. We would therefore consider this feature as an intrinsic property of the $P2Y_4$ receptor, not requiring the transfection of auxiliary proteins in order to form oligomeric structures. Moreover, a particularity of the $P2Y_4$ receptor is the absence of any potential N-glycosylation site. Since glycosylation is a post-translational modification common to many GPCRs involved in certain cases in the regulation of receptor surface expression and dimerisation [29], its potential participation in the control of $P2Y_4$ oligomerisation can be likely ruled out.

Different authors often reported apparently conflicting molecular masses for the $P2Y_4$ receptor [22, 30]. It was often postulated that the reason for this variability could rely on post–translational modifications, diverse among different tissues, such as glycosylation. With our work, we now demonstrate that different aggregation states could also account for the higher-order molecular masses reported for the $P2Y_4$ receptor.

Totally new within the P2Y class, our results instead confirm what is already established for other GPCRs, which can directly associate as either homo- or hetero-oligomers, altering their respective pharmacology and sustaining, in this way, different functions from that evoked by the original receptors. For example, it is well documented that adenosine A1 and A2A receptors form homodimers [24, 31, 32], that these same subunits heterodimerise with dopamine D1 and D2 receptors [33–35] or with metabotropic glutamate receptors (mGluR) [36, 37]. Specifically within the purinergic class, co–localisation at the membrane level between the metabotropic $P2Y_4$ and the ionotropic $NMDAR_1$, and their functional interplay occurring during glucose starvation [17] was recently demonstrated in CGNs and human neuroblastoma cells. Moreover, the $P2Y_1$ subunit is able to form heteromeric complexes with the adenosine A1 subtype [24–26], generating an adenosine receptor with P2Y-like agonistic pharmacology. As a consequence, adenosine 5′-O-(2-thiodiphosphate), a potent $P2Y_1$ agonist, binds to the A_1 pocket of the $A_1/P2Y_1$ complex, inhibiting adenylyl cyclase activity via Gi/o protein and, in this way, the hetero-oligomerisation between adenosine and P2Y receptors might be one of the mechanisms for the adenine nucleotide-mediated inhibition of neurotransmitter release [38]. Since these mechanisms further generate functional and structural heterogeneity among P2 receptors, they certainly contribute to the explanation of how different cells can evoke diverse responses in different tissues upon the same kind of agonist stimulation. For example, it appears from the literature that the ATP effect on the human $P2Y_4$ receptor in transfected cells is variable. ATP has been actually described either as a partial agonist with antagonistic properties [39] or a full antagonist [40]. Whereas some of the discrepancies have been ascribed to the different receptor expression level in different transfected cell lines, in light of our results, we can now hypothesise that the heterogeneous behaviour exerted by ATP could also rely on the proportion of $P2Y_4$ oligomerisation.

Under this perspective, it is therefore noteworthy to have established that the $P2Y_4$ subtype can retain at least homo-oligomeric structures, both in native and transfected cell systems. The main physiological relevance of this investigation will come from studies regarding the exact sub–cellular localisation of the oligomeric forms of the receptor. This and analysis of the possibility that additional P2Y receptors might undergo homo- or hetero–multimeric assembly are therefore our next goals.

In conclusion, since the composition of the oligomers profoundly affects the biological response of P2 receptors and different subtype combinations thus yield different receptor characteristics, allowing diversity in agonists and antagonists selectivity, transmission signalling, channel and desensitisation properties, we do believe that the quaternary structure of the $P2Y_4$, among other probable P2Y receptors, might in part contribute to explain how P2 metabotropic subtypes can trigger so many purinergic functions.

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