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Comparative analysis of $P2Y_4$ and $P2Y_6$ receptor architecture in native and transfected neuronal systems

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Abstract

Although extensive studies provided molecular and pharmacological characterization of metabotropic P2Y receptors for extracellular nucleotides, little is still known about their quaternary structure. By the use of transfected cellular systems and SDS-PAGE, in our previous work we established the propensity of $P2Y_4$ receptor to form dimeric interactions. Here we focused on endogenously expressed $P2Y_4$ and $P2Y_6$ subtypes, comparing their oligomeric complexes under Blue Native (BN) gel electrophoresis. We provided evidence that $P2Y_4$ and $P2Y_6$ receptors form high order complexes in native neuronal phenotypes and that the oligomers can be disaggregated down to the dimeric $P2Y_4$ or to the dimeric and monomeric $P2Y_6$ receptor. Moreover, dimeric $P2Y_4$ and monomeric $P2Y_6$ proteins display selective microdomain partitioning in lipid rafts from specialized subcellular compartments such as synaptosomes. Ligand activation by UTP shifted the oligomerization of $P2Y_6$ but not of $P2Y_4$ receptor, as analysed by BN electrophoresis. Finally, whereas transfected $P2Y_4$ and $P2Y_6$ proteins homo-interact and posses the appropriate domains to associate with all $P2Y_{1,2,4,6,11}$ subtypes, in naive PC12 cells the endogenous $P2Y_4$ forms hetero-oligomers only with the $P2Y_6$ subunit. In conclusion, our results indicate that quaternary structure distinguishing $P2Y_4$ from $P2Y_6$ receptors might be crucial for specific ligand activation, membrane partitioning and consequent functional regulation.

Keywords: GPCR; Purinergic receptor; Dimerization; Lipid raft; Synaptosomes

1. Introduction

Whereas G protein-coupled receptors (GPCRs) have been depicted for a long time as monomeric subunits interacting with hetero-trimeric G proteins upon ligand activation, more recently this vision was unsettled by a growing body of biochemical and biophysical evidence indicating that the propensity to form homo- and hetero-dimers is widespread also in the GPCR family [1–3]. Several studies mainly performed with recombinant proteins have indeed shown that dimerization occurs early after receptor biosynthesis, suggesting either a primary role in receptor maturation and transport to the plasma membrane, or a more general requisite for function [4].

P2Y metabotropic receptors, widely distributed in the central and peripheral nervous system and involved in various functions ranging from neurotransmission and neuromodulation to trophic and toxic actions [5], belong to the superfamily of GPCRs. They are categorized into a subfamily that predominantly couple to Gq protein and activate phospholipase C-β ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptor), and into a subfamily of Gi protein-coupled receptors that inhibit adenylyl cyclase and regulate ion channels ($P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors) [6]. Although extensive studies provided molecular and pharmacological characterization of P2Y GPCRs [7], little is still known particularly about their quaternary structure. On this subject, recent studies demonstrated that the $P2Y_1$ subunit forms constitutive and agonist-promoted hetero-complexes with adenosine A_1 receptor, as shown by co-immunoprecipita-

Abbreviations: BN, Blue Native; DM, N-dodecyl-β-maltoside; DTT, dithiothreitol; FCS, foetal calf serum; GPCRs, G protein-coupled receptors; HDAC, histone deacetylase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; SNAP, synaptosome-associated protein

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tion and co-localization experiments performed both in transfected cells and in rat brain [8–10]. Moreover, it was demonstrated by fluorescence energy transfer measurements that the transfected $P2Y_2$ receptor forms oligomers [11], and that the active metabolite of the antagonist Clopidogrel specific for the $P2Y_{12}$ receptor can modulate protein oligomerization in platelets *in vivo* and in full-length $P2Y_{12}$-expressing HEK cells [12].

By the use of transfected cellular systems and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), in our previous work we established the propensity of $P2Y_4$ receptor to form dimeric complexes partially sensitive to reducing agents [13]. Since caveats must be generally placed on the analysis of immunoprecipitation studies using transfected proteins, in the present work we focused our analysis on endogenously expressed GPCRs, evaluating the quaternary structure particularly of native $P2Y_4$ and $P2Y_6$ subtypes. This choice was justified by the notion that, within the same subfamily of Gq-coupled P2Y proteins, these two receptors display 40% aminoacid homology [14], are expressed at comparably moderate levels in the central nervous system [15], and are specifically activated by pyrimidine nucleotides [7]. Nevertheless, they are differently regulated by agonists, desensitised and recycled at the plasma membrane [16]. With our present work, we highlighted additional differences between these two receptors, and provided biochemical evidence that whereas they both can form high order homo- and/or hetero-oligomers, the protomeric unit at the basis of each complex appeared to be the monomer with respect to the $P2Y_6$ receptor, or the dimer for the $P2Y_4$ subtype. Moreover, the monomeric/dimeric protomers are differently distributed in specialized membrane microdomains such as lipid rafts from cerebellar synaptosomes, and the $P2Y_4$ and $P2Y_6$ oligomeric complexes are differently stabilized by agonist activation.

In synthesis, our results represent a further step in the molecular knowledge of GPCRs and particularly of $P2Y_4$ and $P2Y_6$ receptors, prospecting novel pharmacological and functional consequences related to their complex oligomerization.

2. Materials and methods

2.1. Cell lines and pharmacological treatments

Rat phaeochromocytoma PC12 cells were cultured on collagen-coated dishes in RPMI 1640 medium (Gibco BRL, MI-Italy) supplemented with 10% heat inactivated horse serum and 5% foetal calf serum (FCS) (Gibco BRL); human neuroblastoma SH-SY5Y cells were maintained in DMEM/F12 medium (Sigma-Aldrich, MI, Italy), added 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. For ligand stimulation experiments, 100 μM UTP (Sigma-Aldrich) was directly added to cells in complete culture media, for the indicated times.

2.2. Construction of epitope-tagged human P2Y receptors and transient transfections

Myc- and FLAG-$P2Y_4$ plasmids were obtained as previously described [13]. cDNAs encoding for $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ were digested with *Eco*RI and *Xho*I restriction enzymes and ligated into a pCMV expression vector that incorporated a FLAG epitope tag (DYKDDDDK) at the 5′ terminus. In addition, by the use of polymerase chain reaction with Platinum® *Pfx* polymerase (Invitrogen, Paisley, UK), all cDNAs were also amplified with appropriate oligonucleotides containing *Eco*RI and *Xho*I restriction sequences for the subcloning of each DNA into a pCS2 plasmid with a c-Myc epitope tag at the 5′ terminus. All constructs were verified by sequencing analysis. SH-SY5Y cells were transiently transfected with P2Y receptor cDNAs individually or in pairs using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions, in serum-free medium (Opti-MEM, Invitrogen) for 24 h. Control cells (mocks) were transfected with the empty pCMV or pCS2 vectors.

2.3. Protein extraction

In order to isolate total protein extracts, cells were harvested with either ice-cold RIPA buffer (PBS, 1% Nonidet P-40—NP-40-, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate—SDS-), or an hypotonic buffer composed of 20 mM Tris–HCl, pH 7.4, 10 mM NaCl, in the absence or presence of 1% Triton-X 100, 1% N-dodecyl-β-maltoside (DM) or 0.1 to 1% digitonin. For Blue Native (BN) gels, cells were lysed in BN-lysis buffer (50 mM Bis-Tris, pH 7, 1% DM, 10% glycerol, 0.75 M aminocaproic acid). Cells were also lysed in buffer G (20 mM Tris, pH 7.4, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA), in order to be immunoprecipitated. All 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 [17].

2.4. Immunoprecipitation

Cell lysates from tagged-receptor transfected cells 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. Native $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor in PC12 cells were immunoprecipitated with 2 μg of anti-$P2Y_2$, anti-$P2Y_4$ or -$P2Y_6$ antisera (Alomone Labs, Jerusalem, Israel) followed by incubation with 25 μl (packed gel) of True Blot™ anti-rabbit IgG beads (eBioscience Inc., San Diego, CA, USA). The immunoadsorbents were recovered by centrifugation for 2 min at 3000×*g* and washed three times by resuspension–centrifugation for 2 min at 3000×*g* in buffer G. The samples were eluted in 30 μl of 2× sample buffer used for SDS- PAGE.

2.5. Synaptosome Triton X-100 solubilization and sucrose floatation gradients

Synaptosomes were obtained from rat cerebella by means of differential centrifugation, essentially as previously described [18]. Briefly, post-nuclear supernatants were centrifuged at 9200×*g* for 15 min to yield a pellet corresponding to partially purified synaptosomes. These pellets (6 mg of proteins) were resuspended in 750 μl of buffer A (150 mM NaCl, 2 mM EGTA, 50 mM Tris–HCl, pH 7.5, protease inhibitors) containing 1% (w/v) Triton X-100. After 30 min on ice, each sample was adjusted to 1.2 M sucrose, placed in a centrifuge tube and overlaid with a linear gradient ranging from 30% to 5% sucrose (all prepared in buffer A). The gradients were centrifuged at 190,000×*g* for 19 h using a rotor SW 41 Ti (Beckman Instruments, Inc., CA, and USA). Fifteen fractions (800 μl each), and the pellets resuspended in 800 μl of buffer A were collected and analysed by means of SDS-PAGE and western blotting. The sucrose concentration in each fraction was determined by refractometry.

2.6. SDS-, BN-PAGE and western blot analysis

Analysis of denatured protein components was performed on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Biosciences, Cologno Monzese, Italy). BN-PAGE was carried out essentially as described [19]: total proteins (in native form or incubated for 1 h at 37 °C with SDS from 0.1 to 2%) were run on 8–16% gradient or 8% or 12% linear gels and blotted on PVDF membranes (Amersham Biosciences). In all cases, after saturation blots were probed overnight at 4 °C, with anti-$P2Y_4$ or anti $P2Y_6$ antisera (Alomone Labs) used 1:300 (specificity tested by immunoreactions