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Flexible subunit stoichiometry of functional human P2X2/3 heteromeric receptors

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ABSTRACT

The aim of the present work was to clarify whether heterotrimeric P2X2/3 receptors have a fixed subunit stoichiometry consisting of one P2X2 and two P2X3 subunits as previously suggested, or a flexible stoichiometry containing also the inverse subunit composition. For this purpose we transfected HEK293 cells with P2X2 and P2X3 encoding cDNA at the ratios of 1:2 and 4:1, and analysed the biophysical and pharmacological properties of the generated receptors by means of the whole-cell patch-clamp technique. The concentration-response curves for the selective agonist α,β-meATP did not differ from each other under the two transfection ratios. However, co-expression of an inactive P2X2 mutant and the wild type P2X3 subunit and *vice versa* resulted in characteristic distortions of the α,β-meATP concentration–response relationships, depending on which subunit was expressed in excess, suggesting that HEK293 cells express mixtures of $(P2X2)_1/(P2X3)_2$ and $(P2X2)_2/(P2X3)_1$ receptors. Whereas the allosteric modulators H^+ and Zn^{2+} failed to discriminate between the two possible heterotrimeric receptor variants, the α,β-meATP-induced responses were blocked more potently by the competitive antagonist A317491, when the P2X2 subunit was expressed in deficit of the P2X3 subunit. Furthermore, blue-native PAGE analysis of P2X2 and P2X3 subunits co-expressed in *Xenopus laevis* oocytes and HEK293 cells revealed that plasma membrane-bound P2X2/3 receptors appeared in two clearly distinct heterotrimeric complexes: a $(P2X2\text{-}GFP)_2/(P2X3)_1$ complex and a $(P2X2\text{-}GFP)_1/(P2X3)_2$ complex. These data strongly indicate that the stoichiometry of the heteromeric P2X2/3 receptor is not fixed, but determined in a permutational manner by the relative availability of P2X2 and P2X3 subunits.

1. Introduction

Extracellular nucleotide receptors belong to the P2X (ligand-gated cationic channels) or P2Y type (G protein-coupled receptors) (Abbracchio and Burnstock, 1994; Ralevic and Burnstock, 1998). Seven distinct P2X receptor subunits have been cloned from mammalian species (P2X1-7); they were found to form both homomeric and heteromeric channels. P2X receptors consist of three subunits (Nicke et al., 1998; Aschrafi et al., 2004; Kawate et al., 2009), each of them exhibiting two transmembrane regions, intracellular N- and C-termini and a large extracellular loop (North, 2002; Köles et al., 2007; Hausmann et al., 2015).

Early studies based on co-immunoprecipitation experiments with epitope-tagged subunits demonstrated that only the P2X6 subunit was not able to form homotrimers, and P2X7 was the only exception of constituting heterotrimeric complexes (Torres et al., 1999; Coddou et al., 2011). Because heteromeric P2X receptors mostly combine the pharmacological and biophysical properties of their parent subunits, the functional diversity of native P2X receptor-mediated responses has been explained by the existence of heteromeric assemblies (Nörenberg and Illes, 2000).

Abbreviations: 2-MeSATP, 2-methylthio ATP; ASIC, acid-sensing ion channel; BN-PAGE, blue native PAGE; DRG, dorsal root ganglion; EC_{50}, half maximal effective concentration; FR, fluorescent ratio; GFP, green fluorescent protein; I_{max}, peak current caused by maximum agonist concentration; LGIC, ligand-gated ion channel; n_H, Hill coefficient; TNP-ATP, trinitrophenyl-ATP; wt, wild type; α,β-meATP, α,β-methylene ATP.

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However, in spite of a large variability in recombinant P2X subunit combinations in expression systems, convincing evidence for native heteromeric P2X receptors is rather limited (Saul et al., 2013). The existence of P2X2/3 receptors has been firmly established in sensory ganglia involved in the transduction of painful stimuli from peripheral tissues to the spinal cord dorsal horn (Lewis et al., 1995; Wirkner et al., 2007). Quantitative real time PCR and pharmacological characterization by whole-cell patch-clamp experiments suggest the presence of P2X1/5 receptors in cortical astrocytes (Lalo et al., 2008) and possibly also at sympathetic neuroeffector junctions in submucosal artieries (Surprenant et al., 2000). Although during neuronal differentiation of neural progenitor cells, the expression of both P2X2 and P2X6 subunits was up-regulated (Schwindt et al., 2011), there is no clear evidence for the presence of functional heteromeric P2X2/6 assemblies in native cells (Saul et al., 2013). In the case of co-expressed P2X4 and P2X7 subunits, the current view is that P2X4 and P2X7 receptors exist as independent homomeric channels interacting with each other (Nicke, 2008; Boumechache et al., 2009; Antonio et al., 2011) rather than as heterotrimeric P2X4/7 channels (Guo et al., 2007; Casas-Pruneda et al., 2009).

For heterotrimeric P2X2/3 receptors in HEK293 cells transfected with a lower amount of P2X2 cDNA than of P2X3 cDNA (P2X2:P2X3 cDNA ratio<0.5) it was concluded that the subunit stoichiometry is $(P2X2)_1/(P2X3)_2$ (Jiang et al., 2003; Wilkinson et al., 2006; Hausmann et al., 2012). Functional analysis of wild type (wt) or mutated heteromeric P2X2/6 receptors in HEK293 cells transfected with an excess of P2X6 encoding cDNA over P2X2 cDNA (P2X2:P2X6 cDNA ratio 0.25) revealed that the functional P2X2/6 channels have a $(P2X2)_2/(P2X6)_1$ stoichiometry (Hausmann et al., 2012). However, an atomic force microscopy study suggested that the subunit stoichiometry of the P2X2/6 receptor depends on the relative expression level of the two subunits (Barrera et al., 2007). Thus, in this study the ratio of P2X2 and P2X6 cDNA during the transfection procedure appeared to determine the relative amount of expression of the simultaneously occurring $(P2X2)_1/(P2X6)_2$ and $(P2X2)_2/(P2X6)_1$.

Based on these findings we decided to investigate whether human (h)P2X2/3 receptors have a fixed subunit composition in which hP2X3 predominates over hP2X2 or whether the reverse stoichiometry is also feasible. For this purpose we modified the hP2X2 and hP2X3 cDNA ratios when transfecting HEK293 cells. The patch-clamp method allowed the functional discrimination of two co-existing heteromeric receptor variants. Furthermore, blue native (BN)-PAGE analysis of hP2X2 and hP2X3 subunits co-expressed in *Xenopus laevis* oocytes revealed the simultaneous presence of heterotrimeric $(hP2X2)_1/(hP2X3)_2$ and $(hP2X2)_2/(hP2X3)_1$ protein complexes in their plasma membranes. In conclusion, we suggest that hP2X2/3 receptors have a variable rather than a fixed subunit stoichiometry.

2. Methods

2.1. Cell culture, mutagenesis and transfection procedures in HEK293 cells

HEK293 cells were kept in Dulbecco's modified Eagle's medium additionally containing 4.5 mg/ml D-glucose, 10% fetal bovine serum (Biochrom) and 2 mM L-glutamine (Life technologies) at 37 °C and 5% CO_2 in humidified air.

The hP2X2 ($hP2X2_A$) and the hP2X3 receptor cDNAs were subcloned into pIRES2-DsRed ($hP2X2_A$) and pIRES2-EGFP (hP2X3) vectors from Clontech by *PstI* and *EcoRI* restriction sites (Bodnar et al., 2011). hP2X2 and hP2X3 replacement mutants were generated by QuikChange site directed-mutagenesis (Stratagene).

HEK293 cells were plated 6 h before transient transfection in plastic dishes and glass coverslips for electrophysiology and Ca^{2+} microfluorometry, respectively. For transfection 0.5 μg (homomeric hP2X2 and hP2X3 receptors, heteromeric hP2X2/hP2X3 receptors and heteromeric hP2X2/hP2X3-mut receptors) to 0.75 μg (heteromeric hP2X2-mut/hP2X3 receptors) of plasmid DNA was combined with 10 μl of PolyFect reagent (Qiagen) and 100 μl of OptiMEM (Life technologies). About 18 h after transfection, the medium was replaced by OptiMEM for removal of residual plasmid DNA, as previously described (Kowalski et al., 2014).

2.2. Whole-cell patch-clamp recordings in HEK293 cells

Whole-cell patch-clamp recordings were performed 2–4 days after transient transfection of HEK293 cells at room temperature (20–22 °C) using an Axopatch 200 B patch clamp amplifier (Molecular Devices). Transfected HEK293 cells were identified by means of an inverted differential interference contrast microscope with epifluorescent optics (Axiovert 100, Zeiss). The pipette solution contained (in mM) CsCl 135, $MgCl_2$ 2, HEPES 20, EGTA 11, $CaCl_2$ 1, Mg-ATP 1.5, and Li-GTP 0.3; pH adjusted to 7.4 with CsOH. When 2-methylthio ATP (2-MeSATP) was used as an agonist, Li-GTP was replaced by GDP-β-S (0.3 mM) in order to block G protein-coupled P2Y receptors, which might interfere with P2X receptor mediated responses (Gerevich et al., 2007). The external physiological solution contained (in mM) NaCl 135, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, and D-glucose 11; pH adjusted to 7.4 with NaOH.

The pipette resistances varied between 3 and 7 MΩ. The liquid junction potential (V_{LJ}) between the bath and pipette solution at 21 °C was calculated and was found to be −4.5 mV. Holding potential values given in this study were corrected for V_{LJ}. All recordings were carried out at a holding potential of −65 mV. Data were filtered at 2 kHz with the inbuilt filter of the amplifier, digitized at 5 kHz, and stored on a computer using a Digidata 1440 interface and pClamp 10.2 software (Molecular Devices).

Drugs were dissolved in the external solution and locally superfused to single cells using a rapid solution exchange system (SF-77B Perfusion Fast-Step, Warner Instruments). Concentration-response curves were constructed by applying increasing concentrations of the P2X1,3-selective agonist α,β-methylene ATP (α,β-meATP) or the non-selective P2X agonist 2-MeSATP (0.3–300 μM each; both from Tocris) for 2 s. In all further experiments, stable concentrations (30 μM) of α,β-meATP or 2-MeSATP were applied repeatedly. The intervals between applications were kept at 2 min (hP2X2, hP2X2/3) or 5 min (hP2X3), in order to avoid desensitization. Under these conditions, agonist responses were reproducible (for hP2X3 receptors see Suppl. Fig. 1C).

In experiments analysing the effects of modulators or antagonists, low pH external solutions as well as increasing concentrations of Zn^{2+}, TNP-ATP and A317491 (all from Sigma–Aldrich) were superfused 2–5 min before (depending on the inter-agonist interval) and during α,β-meATP or 2-MeSATP application.

2.3. Ca^{2+} microfluorimetry in HEK293 cells

HEK293 cells were loaded 2–3 days after transient transfection, with the Ca^{2+} sensitive fluorescent dye Fura-2 acetoxymethyl ester (Fura-2/AM; 2.5 μM; Sigma–Aldrich) at 37 °C for 1 h in culture medium. Cells plated onto coverslips were mounted into the superfusion chamber and placed on the stage of an inverted microscope (IX-70; Olympus) with epifluorescence optics and a cooled CCD camera (IMAGO; Till Photonics). Throughout the experiments, cells were continuously superfused at 0.8 ml/min by means of a roller pump with external solution. Intracellular Fura-2 was alternately excited at 340 and 380 nm, and the emitted light