

AGONIST-INDUCED HOMO-DIMERISATION OF P2Y₁ RECEPTORS DEMONSTRATED BY FLUORESCENCE RESONANCE ENERGY TRANSFER ANALYSIS

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A number of G-protein-coupled receptors (GPCRs) of the rhodopsin-like class A have been reported to form homo- or hetero-dimers, but there is as yet no consensus on whether this is general in that class, or constitutive, nor on the effect of agonists in this interaction. We have investigated this for the P2Y receptors for ATP, reporting here on the P2Y₁ subtype. We employ the fluorescence resonance energy transfer (FRET) technique, which detects with high sensitivity the energy transfer between two fluorophores when they are held in close proximity (< 10 nm) in a protein assembly.

The human P2Y₁ receptor was tagged at its N-terminus with the Myc epitope and stably expressed in 293 cells. It was reacted in the intact cells with anti-Myc antibody directly conjugated to fluorescein (FRET donor) or to Cyanine 3 (Cy3; FRET acceptor) or was reacted with both of those labelled antibodies to about equal labelling. In assays on such cells using the intracellular release of Ca²⁺ evoked by the P2Y₁ agonist 2-methylthio-ADP (2-MeSADP), EC₅₀ was not affected by the Myc tag nor by the binding of either antibody. In confocal microscopy both antibody-labelled receptors were localised essentially on the cell membrane and measurements were made on that zone alone. When receptors labelled with a donor or acceptor fluorophore were both present the emission spectrum of the acceptor became modified, denoting FRET. The FRET efficiency could be calculated from the decay rate of donor photobleaching as retarded by acceptor interaction and hence, using the relative affinities of the two antibodies on the receptor, the fraction of dimer formation (1). Without agonist, the P2Y₁ receptors readily showed a 21.2±3.4% (n=3) basal level of dimer formation. With 35 min exposure to saturating concentration of agonist (10 μM 2-MeSADP) at 20°C, the dimer formation increased to a maximum of 87.3±4.2% (n=3). At 37°C, after 20 min treatment the same maximum dimer formation was reached. There was no significant difference in this maximum of dimer formation when the reaction with the labelling antibody was made before or after the agonist treatment, i.e. the bound antibody did not change the dimerisation. Agonist-promoted dimerisation was concentration-dependent and could be fully blocked by the P2Y₁-specific antagonist MRS 2179.

As an independent confirmation, in dual-labelled cells pre-treated with 2-MeSADP (10 μM, 20 min, at 37°C) and with prolonged excitation of the Cy3 acceptor at 543nm, which diminished the emission peak of Cy3 in wavelength scans, that of FITC (initially decreased by the FRET) was progressively augmented by the acceptor photobleaching. Overall, the approach used here has the advantage that it is performed on intact, living cells, unlike other dimer measurements requiring cell disruption. Only receptors on the cell membrane register in it, an important consideration since in cell lines receptors in general have an intracellular fraction, whose local density and association state may differ from that of the mature receptors at the cell surface.

We conclude that the P2Y₁ receptor at the cell membrane normally exists largely as a monomer, but it becomes almost fully dimerised on longer agonist exposure. That change is obviously far slower than the signalling reactions of the receptor and we propose that it is a prelude to its internalisation and turnover.

Gadella, T.W.J. and Jovin T.M. (1995). *J. Cell. Biol.* **129**, 1543-1558.

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