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A membrane network of receptors and enzymes for adenine nucleotides and nucleosides

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ABSTRACT

Most cells express more than one receptor plus degrading enzymes for adenine nucleotides or nucleosides, and cellular responses to purines are rarely compatible with the actions of single receptors. Therefore, these receptors are viewed as components of a combinatorial receptor web rather than self-dependent entities, but it remained unclear to what extent they can associate with each other to form signalling units. $P2Y_1$, $P2Y_2$, $P2Y_{12}$, $P2Y_{13}$, $P2X_2$, A_1, A_{2A} receptors and NTPDase1 and -2 were expressed as fluorescent fusion proteins which were targeted to membranes and signalled like the unlabelled counterparts. When tested by FRET microscopy, all the G protein-coupled receptors proved able to form heterooligomers with each other, and $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$, A_1, A_{2A}, and $P2X_2$ receptors also formed homooligomers. P2Y receptors did not associate with P2X, but G protein-coupled receptors formed heterooligomers with NTPDase1, but not NTPDase2. The specificity of prototypic interactions ($P2Y_1$/$P2Y_1$, A_{2A}/$P2Y_1$, A_{2A}/$P2Y_{12}$) was corroborated by FRET competition or co-immunoprecipitation. These results demonstrate that G protein-coupled purine receptors associate with each other and with NTPDase1 in a highly promiscuous manner. Thus, purinergic signalling is not only determined by the expression of receptors and enzymes but also by their direct interaction within a previously unrecognized multifarious membrane network.

1. Introduction

Extracellular adenine nucleosides and nucleotides are involved in a plethora of physiological and pathophysiological functions such as muscle contraction, platelet aggregation, cell proliferation, chemosensory signalling, immune and inflammatory responses, and neurotransmission. These actions are mediated by two separate families of membrane receptors named P1 and P2. P1 receptors are those for adenosine and can be subdivided in A_1, A_{2A}, A_{2B}, and A_3 receptors all of which are heptahelical receptors typically coupled to heterotrimeric GTP binding proteins [1,2]. P2 receptors comprise two different subgroups: P2X receptors are ATP-gated cation channels composed of three out of a set of at least 7 different subunits [3]; P2Y receptors are heptahelical G protein coupled receptors (GPCRs) and at least 8 different subtypes have been identified so far ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) [4]. In addition, a separate class of receptors has been proposed to exist and to accept nucleosides as well as nucleotides as agonists [5,6], and this class was suggested to be named P3 [7]. However, the existence of this latter type of receptors has not been confirmed by molecular means.

Most of the receptors mentioned above display a rather widespread distribution in mammals. In particular, A_1 and A_{2A}, as well as $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{13}$, and $P2Y_{14}$ receptors are expressed in quite a number of different tissues, whereas the other receptors show a more restricted expression pattern [2,4]. P2X receptors, for example, are confined to excitable tissues and some hemopoietic and epithelial cells (North, 2002). Thus, most mammalian cells do express by far more than one of the receptors that are activated by adenine nucleosides or nucleotides. Moreover, the functional responses to various nucleotides and nucleosides as observed in many tissues are not entirely compatible with the pharmacological characteristics of a single cloned receptor [8]. Therefore, Volonte et al. [9] suggested to regard single receptors for nucleotides as part of a "combinatorial receptor web".

Extracellular nucleosides and nucleotides are found in virtually all types of tissues as they are released from a large variety of cells. One obvious reason for the presence of nucleosides and nucleotides in the extracellular space is cell damage. In addition, release of adenosine may occur through various transporters present in the plasma membrane [2]. Nucleotides, in contrast, are released either through vesicle exocytosis from neurons and neuroendocrine cells or via largely unidentified mechanisms from all other types of cells [10]. However, the extracellular concentrations of adenosine, AMP, ADP, and ATP do not only depend on the release of each of the various species, but are also regulated by various enzymes which mediate the

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stepwise hydrolysis of nucleoside triphosphates towards nucleosides in the extracellular space. The initial reactions are provided by families of ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-nucleotide pyrophosphatase/phosphodiesterases some of which are integral membrane proteins. The final step leading to nucleosides is mainly catalyzed by ecto-5′-nucleotidase which is either membrane-associated via a GPI anchor or soluble. Like P1 and P2 receptors, these enzymes display a rather widespread expression pattern and most cells harbor more than one representative [11].

In many tissues, the actions of endogenous nucleotides are indirect ones and involve not only receptors, but also degrading enzymes. In hippocampal neurons, for instance, adenine nucleotides are rapidly converted to adenosine which activates inhibitory A_1 receptors [12]. In hepatoma cells [13], ATP is degraded to ADP to stimulate $P2Y_1$ receptors. In osteoblastic [14] and epithelial [15] cells, however, ATP itself activates $P2Y_2$ and $P2Y_{11}$ receptors, respectively. In neuroendocrine cells, direct and indirect effects of ATP occur in parallel via $P2Y_2$ and $P2Y_{12}$ receptors, respectively [16]. Hence, a localization of enzymes in close proximity to certain receptors can be expected to underlie indirect effects of nucleotides.

In order to provide a basis for the proposed “combinatorial receptor web” [9] as well as for enzyme-dependent indirect nucleotide effects, we investigated the possibilities of associations between different P1 and P2 receptors as well as membrane NTPDases by FRET microscopy. The results reveal that a large number of combinations may occur to form a membrane network of receptors and enzymes for adenine nucleosides and nucleotides.

2. Materials and methods

2.1. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells, tsA 201 cells (a subclone of HEK 293 cells stably expressing the SV40 large T-antigen), and human astrocytoma 1321N1 cells were cultured in Dulbecco's modified Eagle's medium (PAA Laboratories, Pasching, Austria) containing 1 g l^{-1} glucose and L-glutamine, supplemented with 10% fetal bovine serum (PAA) and 25,000 IU l^{-1} penicillin and 25 mg l^{-1} streptomycin (Sigma, Vienna, Austria). For NTPDase activity measurements, cells were seeded in uncoated 24 multiwell plates (about 25,000 cells per well). For Ca^{2+} fluorescence determinations, cells were plated in 96 well plates (about 8000 cells per well) coated with bovine gelatine. For patch-clamp experiments, cells were plated in 35 mm culture dishes coated with rat tail collagen as described [17]. For FRET microscopy, cells were plated on poly-D-lysine-coated glass cover slips. All cells were transfected using the ExGen 500 reagent (Fermentas; St.Leon-Rot, Germany) according to the manufacturer's recommendation. 3 μg DNA in total were transfected and FRET measurements were carried out 24 h later.

$P2X_2$-CFP/-YFP were a kind gift of Dr. Florentina Soto (Seattle, USA), A_1-CFP/-YFP were kindly donated by Dr. Rafael Franco (Barcelona, Spain; [18]), and A_{2A}-CFP/-YFP, FLAG-tagged and non-tagged A_{2A} by Dr. Oliver Kudlacek (Vienna, Austria). To generate fusion proteins of rat P2Y receptors with CFP (P2Y-CFP) at their C-termini, DNA fragments encoding the full length P2Y receptors were obtained using Taq polymerase (Fermentas). The forward primers were used to add an XhoI restriction site upstream of the start codon, and the reverse primer omitted the stop codon and added an EcoRI site. The PCR amplification products were digested with the corresponding restriction enzymes and ligated in-frame in the pECFP-N1 vector (Becton Dickinson, Heidelberg, Germany). A plasmid containing eNTPDase1 fused to CFP and YFP at the N-terminus was constructed using PCR amplification of the full length cDNA. The forward primer was used to introduce an XhoI site and omit the start codon, and the reverse primer added an EcoRI site. The PCR product was ligated in-frame in the pECFP-C1 and pEYFP-C1 vector. NTPDase2 was cut out of the pEGFP-C1 vector with BglI and XhoI and ligated in-frame in vector pEYFP-C1 with the same restriction sites to create NTPDase2 fused to YFP at the N-terminus. cDNAs for rat eNTPDase1 and eNTPDase2 were kindly provided by Herbert Zimmermann (Frankfurt, Germany), for rat P2Y1 by Georg Reiser (Magdeburg, Germany), for rat $P2Y_2$ by Tania Webb (Leicester, UK), for rat $P2Y_{12}$ by Eric Barnard (Cambridge, UK). The rat $P2Y_{13}$ sequence was obtained from PC12 cells by RT-PCR using primers that were predicted to amplify sequences that are identical or similar to regions of identity in the human and mouse $P2Y_{13}$ DNAs. The PCR products with appropriate lengths (951 and 800 bp, respectively) were sequenced and found to be identical with a sequence predicted to code for rat $P2Y_{13}$ (XM-227178) which was corroborated later by expression cloning [19]. The integrity of all generated constructs was verified by sequence analysis.

2.2. Determination of NTPDase activity

NTPDase activity was determined using a Malachite Green Phophatase Assay Kit (BioAssaySystems, Hayward, CA, USA) according to the manufacturer's recommendations. Either untransfected tsA 201 cells or cells transiently expressing untagged or YFP-tagged NTPDase1 or -2 were used for these experiments 48 h after transfection. Cells were incubated in a buffer (in mM: NaCl 150, KCl 4, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES 10) containing 30 μM ATP, 30 μM ADP, or no nucleotides for 10 min at 36 °C. Then, the supernatant was withdrawn and an aliquot (160 μl) was mixed with the malachite green reagent (40 μl) to assay for released phosphates. The optical densities of these mixtures were quantified at a wavelength of 620 nm using 96 well plates positioned in a microplate reader (Victor3, PerkinElmer, Waltham, MA, USA). From these optical density values, nucleotide concentrations were calculated using a calibration curve obtained with dilutions of a phosphate standard solution.

2.3. Determination of cyclic AMP

The accumulation of cyclic AMP was determined in HEK 293 cells 24 h after transfection of A_{2A} receptors by a technique described in detail elsewhere [20]. After incubation in medium containing 2.5 μCi ml^{-1} tritiated adenine for 12 h, the medium was replaced by a buffer (120 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM glucose, 10 mM HEPES, adjusted to pH 7.4 with NaOH) containing 100 μM of the phosphodiesterase inhibitor Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one] and 1 U ml^{-1} adenosine deaminase. Dishes were then kept at room temperature for about 90 min. The A_{2A} receptor agonist CGS 21680 was added and the cultures were incubated for additional 15 min at room temperature. Finally, the buffer was replaced by 1 ml of 2.5% perchloric acid containing 100 μM non-labelled cyclic AMP followed by a 20 minute incubation at 4 °C.

In experiments addressing the signalling of $P2Y_{13}$, 1321N1 astrocytoma cells were transfected with plasmids coding for human β_2 adrenoceptors (provided by M. Lohse Wuerzburg), on one hand, and for untagged or YFP-tagged $P2Y_{13}$ receptors, on the other hand (200 ng each per well). Loading with tritiated adenine and subsequent manipulations were performed as above. During the last 15 min, the buffer contained 10 μM of the β adrenergic agonist isoproterenol, in the absence or presence of 10 nM ADP.

Cyclic AMP was separated from the other purines by a two column chromatographic procedure as described [20]. One tenth of each sample obtained as described above was used for the determination of the total radioactivity. The remaining 900 μl was neutralised by addition of 100 μl 4.2 M KOH and applied to Dowex 50 columns (AG 50W-X4; Bio-Rad, Vienna, Austria) which were then rinsed with 3 ml H_2O. The eluate obtained by the subsequent application of 8 ml H_2O was directly poured onto alumina columns (Bio-Rad, Vienna, Austria), which were then washed with 6 ml H_2O. Finally, cyclic AMP was eluted with 4 ml imidazole buffer (20 mM imidazole in 0.2 M NaCl;