Co-localization and functional cross-talk between A1 and P2Y1 purine receptors in rat hippocampus

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Keywords: adenosine, ATP, brain damage, electron microscopy, G protein coupled receptors, immunocytochemistry

Abstract

Adenosine and ATP, via their specific P1 and P2 receptors, modulate a wide variety of cellular and tissue functions, playing a neuroprotective or neurodegenerative role in brain damage conditions. Although, in general, adenosine inhibits excitability and ATP functions as an excitatory transmitter in the central nervous system, recent data suggest the existence of a heterodimerization and a functional interaction between P1 and P2 receptors in the brain. In particular, interactions of adenosine A1 and P2Y1 receptors may play important roles in the purinergic signalling cascade. In the present work, we investigated the subcellular localization/co-localization of the receptors and their functional cross-talk at the membrane level in Wistar rat hippocampus. This is a particularly vulnerable brain area, which is sensitive to adenosine- and ATP-mediated control of glutamatergic transmission. The postembedding immunogold electron microscopy technique showed that the two receptors are co-localized at the synaptic membranes and surrounding astroglial membranes of glutamatergic synapses. To investigate the functional cross-talk between the two types of purinergic receptors, we evaluated the reciprocal effects of their activation on their G protein coupling. P2Y1 receptor stimulation impaired the potency of A1 receptor coupling to G protein, whereas the stimulation of A1 receptors increased the functional responsiveness of P2Y1 receptors. The results demonstrated an A1–P2Y1 receptor co-localization at glutamatergic synapses and surrounding astrocytes and a functional interaction between these receptors in hippocampus, suggesting ATP and adenosine can interact in purine-mediated signalling. This may be particularly important during pathological conditions, when large amounts of these mediators are released.

Introduction

ATP and adenosine, via their specific P2 and P1 purinergic receptors (Fredholm et al., 1994), mediate a wide variety of physiological processes including neuromodulation and neurotransmission. Moreover, the purinergic system has been implicated in many pathological and neurodegenerative conditions, in which massive release of ATP and, in turn, ADP and adenosine production, occur from damaged or dying cells, i.e. following ischemia, necrosis or trauma (Rathbome et al., 1999; Burnstock, 2004). Several reports have described a dualistic neuroprotective-neuromodulatory role of ATP interacting with the specific ionotropic receptors (P2XR) and G protein coupled receptors (GPCRs; P2YR) (Fredholm et al., 1994). Among P2YR, P2Y1 receptors (P2Y1R) appear to be of particular interest in pathophysiological mechanisms with both detrimental and beneficial effects (Franke & Illes, 2006). On the other hand, through the activation of the inhibitory A1 adenosine receptor (A1R) coupled to G proteins (Dunwiddie & Masino, 2001), adenosine inhibits the release of excitatory neurotransmitters and decreases neuronal excitability, exerting a neuroprotective role (Wardas, 2002).

Recent data have provided evidence for the existence of an association between A1R and P2Y1R. In a co-transfected cell line model these receptors interact directly (Yoshioka & Nakata, 2004) to generate a hetero-oligomer, which has novel pharmacological and functional characteristics indicating a potential role in the purinergic signalling cascade. A1R and P2Y1R have been suggested to be involved in the modulation of brain damage and contribute, alone or in combination, to neuro-degenerative/-regenerative processes (Neary et al., 2003; Franke & Illes, 2006; Von Kugelgen, 2006). Moreover, co-localization of these receptors has been demonstrated by immunofluorescence and immunoprecipitation (Yoshioka et al., 2002). However, no data are at present available on the precise localization/co-localization of A1 and P2Y1 receptors at cellular and subcellular levels, or on their reciprocal modulation/function interaction in the native brain.

Hippocampus is a brain area with a specific vulnerability to injuries, in particular to ischemia (Harry & Lefebvre d’Hellencourt, 2003). In the hippocampus, A1R and P2Y1R are particularly abundant (Gottlieb & Matute, 1997; Ochiishi et al., 1999; Jimenez et al., 2000; Moran-Jimenez & Matute, 2000; Zhu & Kimelberg, 2001) and involved in the modulation of glutamate release (Rudolphi et al., 1992; Mendoza-Fernandez et al., 2000; Masino et al., 2002; Koizumi et al., 2003; Kawamura et al., 2004; Rodrigues et al., 2005; Jourdain et al., 2007), hence contributing to neurotransmission and neuro-degenerative and -regenerative processes.

In the present work, we investigated the localization/co-localization of A1R and P2Y1R in rat hippocampus, focusing on glutamatergic synapses and using electron microscopic (EM) quantification...
of postembedding immunogold labelling; this technique allows the precise localization of proteins to be identified at subcellular resolution, on different parts of astrocytes and neurons.

As a first step to investigate the functional A₁R and P2Y₁R cross-talk, we studied the A₁R activation following P2Y₁R stimulation and vice versa in crude rat hippocampal membranes (HC). For this purpose the [³²S]guanosine-5'-(g-thio)-triphosphate ([³²S]GTPgS) binding assay (Lorenzen et al., 1993, 1996) was used. The GTP binding represents the initial step of any GPCR activation and of the intracellular signalling cascade mediated by GPCRs (Lorenzen et al., 1996).

Materials and methods

Materials

The A₁R antibody was obtained commercially from Alpha Diagnostic International (San Antonio, TX, USA) and the P2Y₁R antibody from Alomone Laboratories (Jerusalem, Israel). The data sheets supplied by the manufacturers showed monospecificity by immunoblotting of rat brain. The A₁R antibody was raised against a 14-amino-acid peptide corresponding to amino acid residues 163–176 of the rat or human receptor protein, i.e. the epitope is in the presumed extracellular N-terminal domain. The P2Y₁R antibody was raised against a 17-amino-acid peptide (C) RALIYKDLNSPLRRKS, corresponding to residues 242–258 of rat or human P2Y₁R, i.e. the epitope location is in the presumed third intracellular loop (3) between the TM5 and TM6 domains. Secondary antibody, goat anti-rabbit IgG-HRP conjugate, was from Calbiochem (EMD Biosciences, affiliate of Merck kgA, Darmstadt, Germany). Goat anti-rabbit immunoglobulins conjugated to 10-nm or 15-nm gold particles were obtained from Aurion (Wageningen, The Netherlands). Secondary antibody, goat anti-rabbit IgG-HRP conjugate, was from Calbiochem (EMD Biosciences, affiliate of Merck kgA, Darmstadt, Germany). Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA, USA); full range rainbow molecular weight markers (range 10–250 kDa) were obtained from Amersham Biosciences (Freiburg, Germany).

[³²S]GTPgS (specific activity 1000 Ci/mmol) was purchased from Amersham Biosciences Europe GmbH (Freiburg, Germany); adenosine deaminase (ADA) was from Roche Diagnostics GmbH (Mannheim, Germany). N⁶-cyclohexyl adenosine (CHA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), GDP, guanosine-5'-(g-thio)triphosphate (GTPₓS), 2'-methylthio-adenosine 5'-diphosphate (MeSADP), 2'-deoxy-N⁶-methyl adenosine 3',5'-diphosphate (MRS2179), and protease inhibitors were obtained from Sigma Chemical Co (St Louis, MO, USA). The protein concentration of the samples was determined using the protein assay based on the Bradford method from Bio-Rad (Hercules, CA, USA), using bovine serum albumin as a standard. All other reagent grade chemicals were supplied from standard commercial sources.

Postembedding immunogold cytochemistry

Immunogold electron microscopy quantification was used to study A₁R and P2Y₁R receptors in rat hippocampus, focusing on glutamatergic synapses. Animals were treated in accordance with the guidelines of the Norwegian Committees on Animal Experimentation (Norwegian Animal Welfare Act and European Communities Council Directive of 24 November 1986-86/609/EEC). Receptor immunocytochemistry was performed as described previously in Bergersen et al. (2005), with some modifications. Wistar rats were deeply anesthetized with an i.p. injection of pentobarbital. Briefly, after transcardiac perfusion with 0.1% glutaraldehyde and 4% formaldehyde in 0.1 M sodium phosphate buffer (NaPi), pH 7.4, the brains from adult male Wistar rats (300 g, n = 3) were left in situ overnight (4 °C). Then hippocampal specimens were isolated, processed by cryoprotection in different glycerol solutions, snap-frozen in liquid propane cooled by liquid nitrogen and embedded in Lowicryl HM 20 through freeze-substitution. Ultrathin sections (80 nm) were cut with a diamond knife on a Reichert-Jung ultramicrotome and mounted on nickel grids (300 mesh square, Electron Microscopy Sciences, USA). The sections were processed at room temperature in solutions of 0.05 M Tris HCl buffer, pH 7.4 containing 0.3% (w/v) for P2Y₁R antibody or 0.1% (for A₁R antibody) NaCl and 0.1% Triton X-100 (TBST) and completed as stated below.

After ‘etching’ in sodium ethanolate to remove plastic from tissues, sections were incubated in TBST containing 2% human serum albumin (HSA) for 10 min and then overnight (around 20 h) with specific primary antibodies diluted in TBST containing 2% HSA. Antibodies against A₁R (dilution 2 μg/mL) as well as the antibody against the P2Y₁R (dilution 4 μg/mL) were used. Sections were then incubated with goat anti-rabbit immunoglobulins coupled to 10-nm gold particles, diluted 1 : 20 in TBST with 2% HSA and, for A₁R experiments, with 2 mg/mL polyethylene glycol to suppress the formation of gold particle aggregates.

The ultrathin sections were processed both with single-labelled and double-labelled procedures. In double-labeling experiments (Ottersen et al., 1992), sections were treated with the antibody against P2Y₁ (dilution as above) in the first step (followed by 10-nm gold-labelled secondary antibody) and then with the A₁R antibody (dilution as above) in the next step (revealed by 15-nm gold-labelled secondary antibody). Exposure to formaldehyde vapours (80 °C, 1 h) was used between the two immunolabelling steps (Wang & Larsson, 1985) to destroy the remaining free anti-IgG binding sites on the first primary and secondary antibodies. Potential cross-reactivity arising from the subsequent use of another secondary antibody that would be directed against the same species is prevented in this manner, allowing the simultaneous detection of two different antigens when using two primary antibodies from the same species, distinguishing the two by means of different gold particle sizes (10 nm for P2Y₁R, 15 nm for A₁R). The double-labelling approaches gave similar patterns as the single-labelling protocol.

Negative control experiments also were performed, replacing the incubation with the primary antibodies with 2% HSA in TBST; staining was absent on sections that had been incubated in such a solution. Ultrathin sections were contrasted in uranyl acetate and lead citrate and observed in a Philips CM100 electron microscope.

Quantitative analysis

Electron micrographs were randomly taken in the hippocampus (magnification 34 000×). Gold particles signalling both receptors (P2Y₁R or A₁R) were quantified as number of gold particles/μm² at glutamatergic synapses (i.e. asymmetric synapses on dendritic spines) in the stratum radiatum of area CA1 and CA3, as well as in the juxtagranular part of the dentate molecular layer. The former are formed by terminals of ipsilateral (Schaffer collaterals) and commissural axon collaterals from CA3 pyramids, the latter by terminals of mossy cells in the dentate hilus. Only synapses with clearly visible postsynaptic membrane and postsynaptic density were selected for analysis; essentially all synapses with this morphology are glutamatergic in these areas (for identification criteria, see Gylterud Owe et al., 2005). Quantitative analyses were carried out on sections single labelled for either P2Y₁R or A₁R. Specific membrane compartments were
defined and used for quantifications; they correspond to the presynaptic vesicles membranes, the membrane overlying the postsynaptic density (PSD), presynaptic membrane (opposite to the PSD), pre- and post-perisynaptic membranes (corresponding to membrane lateral to the presynaptic active zone and the PSD, extending laterally by half the length of the PSD), extrasynaptic membranes (belonging to either presynaptic terminals or postsynaptic spines/dendrites but excluding the synaptic and perisynaptic membranes), postsynaptic intracellular membranes, astroglia membranes and mitochondrial outer membranes (Fig. 1; cf. Bergersen et al., 2005). In addition, gross distribution of gold particles was recorded over the following location categories; presynaptic terminal, postsynaptic spine, synaptic cleft, astrocytes, mitochondria, extrasynaptic membranes, postsynaptic intracellular membranes, astroglia membranes and mitochondrial outer membranes.

Particles located within 40 nm (perpendicular distance between the centre of gold particle and the membrane) from different membranes were recorded. This distance was chosen because 40 nm is approximately equal to the lateral resolution of the immunogold method, i.e. the maximum distance from the centre of the gold particle and the epitope, determined experimentally (Chaudhry et al., 1995; Nagelhus et al., 1998). The areas sampled were determined by grid point analysis (Gundersen et al., 1988; Gundersen et al., 1998), and the densities expressed as gold particles/μm². The distance between the centres of gold particles, representing receptors, and the external face of the postsynaptic density was determined along a perpendicular axis. All gold particles located within the postsynaptic spine and the presynaptic terminal were recorded; the width of the synaptic cleft was also measured. The distribution of gold particles was compared with that of grid points placed randomly over the sampled area (Gundersen, V et al., 1998; Bergersen et al., 2003) in order to relate the distributions of A1R and P2Y₁R to a random distribution.

**Rat brain membrane lysates and Western blotting**

Western blot analysis on rat hippocampus and full-brain membranes has been used to confirm the specificity of the antibodies and the presence of the receptors, as a further control.

The whole brain or the hippocampus were removed from Wistar rats (200–300 g; n = 3) and immediately processed, keeping on ice. The tissues were suspended in 20 volumes of ice-cold 50 mM Tris HCl, 2 mM MgCl₂ buffer, pH 7.4, containing EDTA 1 mM and protease inhibitors (benzamidine 0.16 mg/mL, trypsin inhibitor 0.03 mg/mL and bacitracin 0.2 mg/mL) (buffer A). The tissues were then homogenized with a Polytron homogenizer and after centrifugation (48 000 × g for 10 min at 4 °C), the membrane pellets were resuspended and re-homogenized in buffer A containing ADA 2 U/mL to obtain a concentration of 50 mg/mL (from original tissue weight). After incubation for 30 min at 37 °C, the samples were centrifuged at 4 °C and each pellet was resuspended to the used concentration, boiled in Laemmli solution for 5 min, centrifuged at room temperature for 5 min and the supernatant used for electrophoresis (or kept at −20 °C until use). Tissue membrane homogenates (50 μg) were then processed by immunoblot following the method.

![Fig. 1. Schematic illustration of a synapse between a presynaptic terminal and a dendritic spine. Analysis of gold particle density (number of gold particles/μm²) was performed in specific membrane regions that were defined as presynaptic vesicles membranes, the postsynaptic membrane overlying the postsynaptic density (PSD; red), presynaptic membrane ‘active zone’ (i.e. opposite to the PSD; yellow), pre- and post-perisynaptic membranes on each side of the active zone (blue), extrasynaptic membranes (belonging to either presynaptic terminals or postsynaptic spines/dendrites but excluding the synaptic and perisynaptic membranes; green), postsynaptic intracellular membranes, astroglial plasma membranes (black) and mitochondrial outer membranes (brown). Note that the length of perisynaptic membrane considered corresponds to half the total length of the PSD, on each side (the same is valid for the presynaptic perisynaptic membrane). Figure modified from Bergersen et al. (2005).](https://example.com/synapse_diagram.png)
membranes were also treated with the P2Y1R selective antagonist (control HC) or 100 nM pH 7.4 and then counted in a scintillation cocktail. The concentration of agonist MeSADP was expressed as the per cent increase above the basal unstimulated condition 4.00 and the EC50 values were derived. Data are reported as mean ± SEM; statistical significance refers to results where P < 0.05 was obtained.

Agonist dose–response curves were analysed by the non-linear regression curve-fitting computer program GraphPad PRISM Version 4.00 and the EC50 values were derived. Data are reported as mean ± SEM of four different experiments (performed in duplicate). Statistical analysis (Student’s t-test, two tails, unpaired) was performed using GraphPad PRISM; significance refers to P < 0.05.

### Results

#### Immunolocalization

Postembedding immunogold electron microscopy was used to study A1R and P2Y1R (Fig. 2A and B) in rat hippocampus, focusing on glutamatergic synapses (i.e. small terminals with asymmetric synapses on dendritic spines) and surroundings glia. For quantitative analysis single-labelled sections, were randomly selected from CA1 and CA3 stratum radiatum, and juxtagranular part of the dentate molecular layer, regions that are particularly high in nerve terminal glutamate and glutamatergic markers (e.g. Cotman et al., 1987). As no overt differences were noticed between the areas, they were analysed together. Both A1R and P2Y1R were detected on synaptic and glial membranes (Fig. 2A and B). Omitting the primary antibodies abolished labelling (Fig. 2C), indicating a low unspecific signal due to the detection system.

A1R and P2Y1R antibody specificity was addressed further by Western blot analysis, using rat whole brain and hippocampus membrane fractions (Fig. 2D and E). Results confirmed the presence of the two receptors and their antibody specificity. The A1R immunoreactive band corresponds to the dimer form of A1Rs (79 kDa; Fig. 2D) (Ciruela et al., 1995; Blum et al., 2002), while the P2Y1R multiline pattern shows immunoreactive bands at 76, 40 and 35 kDa, corresponding to dimeric and monomeric variants (Fig. 2E), in agreement with the literature data (Hoffmann et al., 1999; Waldo & Harden, 2004).

The receptor distribution and localization was quantified in different subcellular structures (Fig. 3A and B). The localization of both receptors was expressed as the area density, the number of gold particles/μm², in different structures in the vicinity of hippocampal glutamatergic synapses, determining the area by point analysis (Gundersen et al., 1988). Assuming the mitochondria do not contain the receptors, the density of gold particles over mitochondria may be taken to represent unspecific background binding of antibodies. (This may be an overestimate of background labelling, as the protein concentration in mitochondria is exceptionally high.) Both A1R and P2Y1R were highly concentrated in the synaptic cleft area and in adjacent astrocytes, which were the only compartments significantly higher than mitochondria (Fig. 3A and B). Because the spatial resolution of our immunolabelling method is of the same order of magnitude as the distance between different compartments/membranes in the tissue, an individual gold-particle cannot be directly ascribed to any single structure. Therefore, the association of gold particles with membranes was analysed in several different ways.

First, we investigated the distribution of gold particles associated with each kind of cellular membrane, in the vicinity of hippocampal synapses, recording only particles within a distance equal to 10 nm from each kind of membrane to minimize ‘cross firing’ effects from immunoreactivity in nearby structures. Immunogold single-labelling of hippocampal sections showed that A1R (Fig. 4A) as well as P2Y1R (Fig. 4B) are mainly associated with presynaptic membranes, postsynaptic membranes overlying the PSDs, and astroglial membranes. The high A1R presence on presynaptic-persynaptic membranes (P < 0.05, one-way ANOVA, value vs. mitochondria) could be influenced from receptors that are located in the active zone or as ‘reserve’ (Rebola et al., 2003); anyway the values on persynaptic and extrasynaptic membranes, both pre- and post-, can be influenced from receptors that are instead on astroglia membranes, for the tight contact between these and neurons. No other columns differed from the background level, as indicated by the density of gold particles over mitochondrial outer membranes (P > 0.05, one-way ANOVA). Further, **European Journal of Neuroscience, 26, 890–902**
values at the presynaptic membrane and at the membrane covering the PSD were significantly different compared to all other locations, confirming that A1R are associated with these membranes.

We then compared the distribution of gold particles representing A1R and P2Y1R with the distribution of points spread randomly over the pictures (Fig. 5). The gold particles/random points ratio was determined at different distances, from different membrane domains, sorted into bins (10-20-30-40 nm). For the shortest distance (10 nm) bins, the highest ratio values (approximately 2) were obtained, for both A1R and P2Y1R, at presynaptic active zone membranes, postsynaptic membranes overlying the PSD and astroglial membranes (Fig. 5A and B), i.e. the same membrane domains that show the highest labelling according to the analysis of Fig. 4. Also several other neuronal membranes, but not mitochondrial outer membranes, had ratios higher than one, consistent with a moderate enrichment of receptors in them. As previously noted, the densities in persynaptic and extrasynaptic membranes could be influenced from gold particles in active zone or in astroglial membranes.
Fig. 3. Distribution of immunogold particles indicating A₁R (A) or P₂Y₁R (B) in different intra- and extracellular compartments. Note that both receptors are enriched at synapses and astroglia, compared to all other compartments. Data, from single labelling experiments, report the localization of receptors as the density (gold particles/μm²; mean ± SEM) in the different compartments in the vicinity of hippocampal glutamatergic synapses (i.e. asymmetric synapses on spines, see Materials and methods; randomly selected, from n = 3 animals; five grids analysed for A₁R and six grids for P₂Y₁R); the areas of the compartments were measured by point analysis (see Materials and methods). The numbers of gold particles on each structure are, in order 323, 230, 1, 52, 24, 31, 15 for A₁R; 330, 311, 5, 112, 35, 31, 32 for P₂Y₁R. Areas analysed for each compartment are, in order 10.8, 7.7, 0.1, 1.1, 0.2, 0.6, 1.3, 1.0 μm² for A₁R, 12.1, 8.8, 0.2, 2.1, 0.2, 0.9, 1.7, 0.8 μm² for P₂Y₁R. (A) A₁R. **P < 0.01, one-way ANOVA, all columns compared to mitochondria (Dunnett’s multiple comparison test). Synaptic cleft is significantly different compared to all other domains (#P < 0.01 vs. all except astrocytes, Student’s t-test, two-tails, unpaired) compared to mitochondria. (B) P₂Y₁R; *P < 0.05, **P < 0.01 all domains compared to mitochondria, one-way ANOVA (Dunnett’s multiple comparison test). Synaptic cleft is significantly different compared to all other domains (#P < 0.001 vs. all except astrocytes, One-way ANOVA, Tukey’s multiple comparison test).

Fig. 4. Distribution of immunogold particles indicating A₁R (A) or P₂Y₁R (B) over different categories of cellular membranes in the vicinity of hippocampal synapses. Both receptor types are enriched over presynaptic as well as postsynaptic membranes and over astroglial membranes. Only particles located within a distance equal to 10 nm from each kind of membrane were recorded, to minimize ‘cross firing’ effects from immunoreactivity in nearby structures. The data are presented in the same way, and are from the environments of the same synapses, as are the data in Fig. 3. The sizes of the areas sampled for gold particles (∼10 nm on each side of the membrane) were determined by point analysis. Areas analysed for each category are, in order 3.8, 0.5, 0.3, 1.4, 0.7, 0.3, 1.1, 0.3, 0.6, 0.6 μm² for A₁R; 5.1, 0.8, 0.3, 1.6, 0.8, 0.4, 2.0, 0.6, 1.2, 0.7 μm² for P₂Y₁R. The numbers of gold particles within 10 nm from each membrane column are, in order 196, 126, 22, 98, 141, 19, 54, 32, 57, 12 for A₁R.; 260, 81, 15, 101, 124, 26, 74, 36, 116, 27 for P₂Y₁R. The density of gold particles over the outer membrane of mitochondria gives an estimate of the background level. (A) *P < 0.05, **P < 0.01, all columns compared to mitochondria background level, one-way ANOVA (Dunnett’s multiple comparison test). Presynaptic membrane and PSD are significantly different compared to all other membrane categories (#P < 0.01 vs. all except presynaptic membranes and astroglial membranes, Tukey’s multiple comparison test). (B) **P < 0.01, all columns compared to mitochondria background level, one-way ANOVA (Dunnett’s multiple comparison test). PSD is significantly different compared to all other columns except for presynaptic membranes and astroglial membranes (#P < 0.01, one-way ANOVA, Tukey’s multiple comparison test).
The average distance of immunogold particles from membranes was then measured and compared with a random distribution (Fig. 6). Note that for both A₁R (Fig. 6A) and P2Y₁R (Fig. 6B) the mean distance of gold particles is shorter than that of random points in all membranes, except in mitochondrial outer membranes (which represent an estimate of background labelling).

To dissect further the synaptic localization of A₁R and P2Y₁R, all the hippocampal glutamatergic synapses previously analysed were processed to record the percentage distribution of both receptors as a function of the perpendicular distance from the postsynaptic membrane overlying the PSD (Fig. 7). The percentage distribution of A₁Rs in the synaptic area (Fig. 7A, left) showed that A₁R are located over both the PSD and the presynaptic membrane, although the density is highest over the latter. The influence of the extracellular location of the epitope recognized by the A₁R antibody may have contributed to the high level of gold particles located in the synaptic cleft. A₁R-gold particles situated over the synaptic cleft were preferentially associated with the presynaptic membrane (Fig. 7A, right), indicating a shared distribution of the receptor between the presynaptic and postsynaptic sides. P2Y₁Rs were relatively more distributed towards the PSD membrane (Fig. 7B, left), compared to A₁Rs, and gold particles in the cleft showed less enrichment on the presynaptic side (Fig. 7B, right). These results are consistent with a high degree of co-localization of the two types of receptor, but with relatively less of P2Y₁R than of A₁R on the presynaptic side.

In conclusion, A₁R and P2Y₁R are co-localized in synaptic and astroglial membranes in glutamatergic synapses and surrounding glial membranes in the hippocampus. The quantitative analyses of single-labelling data were confirmed by the qualitative analysis of double-labelling experiments (Fig. 8). The double-labelling approach gave similar patterns as the single labelling protocol; the receptors were detected together in the same structures and both were associated with synaptic and astroglial membranes (Fig. 8).

**Functional assays**

The effect of P2Y₁R activation on A₁R/G protein coupling was measured in HC by evaluating the ability of the A₁R agonist CHA to stimulate G protein activation following 100 nM MeSADP pre-incubation (for 10 min). In control HC, CHA stimulated G protein activation with an EC₅₀ of 30.60 ± 3.99 nM (Fig. 9). To test the CHA-mediated selective A₁R activation in our model, we also stimulated...
HC in the presence of the selective A1R antagonist DPCPX (50 nM; pre-incubated for 10 min). DPCPX prevented CHA-mediated G protein coupling in HC (Fig. 9), showing that the G protein activation is selectively driven by A1R. The CHA-mediated effect was abolished in the presence of the selective A1R antagonist DPCPX (50 nM), confirming A1R specific activation.

Membrane pre-incubation with 100 nM MeSADP induced a right-shift of the CHA dose/response curve (EC50 = 106.26 ± 16.39 nM), suggesting a significant impairment in A1R-G protein coupling when P2Y1 receptor is activated (t-test, two-tails, unpaired P = 0.004 for EC50 of MeSADP-treated HC vs. control HC; Fig. 9).

On the other hand, the A1R activation effect on P2Y1R-G protein coupling was measured by evaluating the ability of the P2Y1R agonist MeSADP to stimulate G protein activation in the absence or the presence of 100 nM CHA (pre-incubated for 10 min). In control HC,
MeSADP stimulated [35S]GTPγS binding with an EC50 of 0.851 ± 0.147 nM (Fig. 10).

To test the selectivity of MeSADP for P2Y1 receptor subtype in our model, we also stimulated HC in the presence of the selective P2Y1R antagonist 10 μM MRS2179 (pre-incubated for 10 min). MRS2179 blocked MeSADP-mediated G protein coupling in HC (Fig. 10), showing this G protein activation is selectively driven by P2Y1R, and other P2Y receptors sensitive to MeSADP (P2Y12-13) have no significant signal component in rat hippocampus. Membrane CHA pre-incubation induced a significant increase in MeSADP potency to activate P2Y1-R-G protein coupling, with an EC50 of 0.393 ± 0.058 nM (t-test; two-tails, unpaired P = 0.03 vs. control HC), without significantly affecting the G protein coupling efficacy level of the agonist dose/response curve (Fig. 10).

Cell treatment with MeSADP or CHA induced an increase in the basal [35S]GTPγS binding value, thus suggesting the agonists affect the G protein activation state. Values are reported as fmol/mg of proteins; control HC = 183.96 ± 30.2; MeSADP HC = 298.65 ± 30.18; CHA

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European Journal of Neuroscience, 26, 890–902
HC = 317.93 ± 8.42 (t-test, two-tails, unpaired, \( P = 0.047 \) for MeSADP-treated HC vs. control HC; \( P = 0.017 \) for CHA-treated HC vs. control HC).

Discussion

Localization and co-localization

This is the first study on A1R and P2Y1R localization and co-localization in hippocampus by postembedding immunogold electron microscopy analysis. This high resolution technique is able to show the precise subcellular localization of receptors in different subcellular compartments and cell populations (e.g. Bergersen et al., 2003). The purinergic receptors proved to be mainly associated with membrane domains. Single-labelling immunolocalization data showed a significant enrichment of both A1R and P2Y1R mainly in postsynaptic membranes at the PSD, in presynaptic active zones, and in astrogial membranes at glutamatergic synapses and surrounding glia in the rat hippocampus. The same conclusion was arrived at by analysing the data in different ways, in order to partly overcome the limits posed by ‘cross firing’ effects antigen located in closely spaced neighbouring membranes. Because of these, the exact labelling densities of the individual membranes cannot be determined. While the three membrane categories mentioned contain higher levels of both of the purinergic receptors studied, low or moderate labelling may exist in other membrane categories. The data suggest that there may be relatively higher densities of A1R than of P2Y1R at the presynaptic compared to the postsynaptic membrane. Part of this observed difference might be attributable to the fact that the antibodies were to extracellular and intracellular epitopes, respectively, although the inner and outer surfaces of the plasma membrane are only 4–5 nm apart, i.e. an order of magnitude less than the lateral resolution of the immunogold method. However, the main conclusion is that the two receptor types are similarly distributed, compatible with a high degree of co-localization. This was born out by double-labelling experiments that showed A1R and P2Y1R to be closely spaced along synaptic and glial membranes.

In parallel, the specificities of A1R and P2Y1R antibodies used in immunohistochemistry, were assessed by immunoblotting assay using rat whole brain and hippocampus membrane fractions. The obtained results were according to literature data (Hoffmann et al., 1999; Blum et al., 2002; Moore et al., 2002; Waldo & Harden, 2004) and confirmed that antibodies selectively recognized A1 and P2Y1 receptors in hippocampal membranes.

Our high resolution data serve to extend and reconcile previous reports obtained with lower resolution methods. Thus A1R immunoreactivity has been reported in hippocampus both at presynaptic and postsynaptic terminals but not at glial cells (Ochiishi et al., 1999); an immunohistochemical study in the rat hippocampus has concluded that A1Rs were mostly located in axons rather than in nerve terminals (Swanson et al., 1995), whereas work on synaptosomal fractions (Rebola et al., 2003) has suggested that A1Rs are enriched in nerve terminals and are mainly located in synapses, both in the presynaptic active zone and in the PSD membranes. P2Y1R immunoreactivity has been found in astroglia and in different kinds of neurons in hippocampus (Moran-Jimenez & Matute, 2000), especially in ischemic sensitive areas while at the same time another study reported a striking neuronal localization for P2Y1R (human brain, Moore et al., 2000). P2Y1Rs have been reported to be present and active on astrocytes all around the brain (Franke et al., 2001; Volonte et al., 2006). A high degree of co-localization of A1R and P2Y1R has been found in rat hippocampus by immunofluorescence experiments but without cellular and subcellular identification (Yoshioka et al., 2002).

We studied A1Rs and P2Y1Rs in the hippocampal region considering the fact that the hippocampus has been identified as a major target site for numerous disease processes (Bachevalier & Meunier, 1996; Harry & Lefebvre d’Hellencourt, 2003), and considering the generally assumed involvement of purinergic receptors in pathophysiological mechanisms and in the modulation of brain damage (Fredholm, 1997; Franke et al., 2006b). Ischemia, to which hippocampus is particularly vulnerable, produces a marked increase in glutamate within the brain extracellular space (Benvensiste et al., 1984; Hagberg et al., 1985), thereby triggering excitotoxic injuries (Choi & Rothman, 1990). Because of the importance of glutamate in pathological conditions and because its release, in neurons and in astrocytes, is modulated both through A1R (Rudolphi et al., 1992; Masino et al., 2002) and P2Y1R (Rodrigues et al., 2005; Franke et al., 2006a; Jourdain et al., 2007), the present study was focused on A1R–P2Y1R localization and co-localization within and in the vicinity of glutamatergic synapses.

Our results provide direct morphological support for the previous suggestions that both of these receptors contribute to and interact in the modulation of glutamate release (Rudolphi et al., 1992; Mendoza-Fernandez et al., 2000; Masino et al., 2002; Kawamura et al., 2004; Rodrigues et al., 2005).

Functional interaction

The functional interaction of A1Rs and P2Y1Rs suggested by the morphological observations was subsequently confirmed through measurement of G protein activation initiated by the A1R agonist, CHA, or the P2Y1R agonist, MeSADP, respectively, and modification of the response through pre-incubation with the other agonist. Because the receptors on study are coupled to different G protein subtypes (Munshi et al., 1991; Yoshioka & Nakata, 2004) and to different intracellular signalling pathways, the \(^{[35S]}\)GTP\(_{\gamma}\)S binding method was chosen to investigate the A1R–P2Y1R interaction and their reciprocal modulation at the membrane level, allowing any change in their functioning to be determined independently of the second messenger systems activated (Lorenzen et al., 1996).

According to literature data (Gao et al., 2003; Dixon et al., 2004; Niebauer et al., 2005), the selected agonist pre-incubation times and concentrations pre-stimulating A1R and P2Y1R (100 nM CHA and 100 nM MeSADP, respectively) allow a selective and maximal receptor activation.

The EC\(_{50}\) for CHA in stimulating A1R-G-protein coupling was around 30 nM. Although CHA has been reported to block \(^{[3]H}\)DPCPX binding at A1R with a \(K_i\) of approximately 5 nM in rat hippocampus (Maemoto et al., 1997), according to our data, higher EC\(_{50}\) values for different A1R agonists in the \(^{[35S]}\)GTP\(_{\gamma}\)S binding assay have been found (Lorenzen et al., 1996; Cordeau et al., 2004). In order to obtain a selective stimulation of A1R, without interference of other receptor subtypes, we have chosen treatment of hippocampal membranes with 100 nM agonist CHA. In fact in hippocampus, although A1Rs are the mainly expressed adenosine receptors, the presence of low amounts of A2A and A3 receptors have been described (Duarte et al., 2006; Lopes et al., 2003). Nevertheless, these receptors have a different agonist pharmacological profile; CHA, in fact, shows a high affinity towards A3Rs (Murphy & Snyder, 1982; Maemoto et al., 1997) and a low affinity towards A2A ones (Cunha et al., 1996; Gao et al., 2003). Even if, in our experimental conditions, the EC\(_{50}\) for CHA in stimulating A1R-G-protein coupling was around 30 nM, the
real binding potency of CHA to A1R sites is around a few nanomolar units or indeed in the subnanomolar range in rat hippocampus, so the agonist at 100 nM is able to selectively saturate A1R binding sites. This was confirmed by an selective dose–response curve, DPCPX was able to completely abrogate the effects mediated by CHA from 1 to 100 nM. At higher agonist concentrations the antagonistic effect of DPCPX was reduced suggesting that the agonist binds to a different receptor population, probably identifiable with A2A receptor sites. On the other hand, in our results, MeSADP showed subnanomolar potency in stimulating P2Y1R. Because of the absolute potency of nucleoside tri- and diphosphates for P2Y receptors is dependent on the levels of receptor protein expression, typical EC50 values are not easily defined for specific agonists at particular P2Y receptor subtypes in different tissue preparations and cell lines (Volonté et al., 2006). In accordance with our results, low nanomolar and subnanomolar EC50 values have been reported for MeSADP towards human P2Y1R, expressed in astrocytoma 1321 N1 cells (Palmer et al., 1998; Niebauer et al., 2005), and rat P2Y1R, expressed in HEK 293 cells (Vörhinger et al., 2000).

MeSADP is the principal agonist not only at P2Y1R but also at P2Y12–13 receptors, that are coupled to Gs proteins and are expressed (mRNA) in the brain (Hollopeter et al., 2001; Zhang et al., 2002; Sasaki et al., 2003), even if not at high level in the hippocampus (Fumagalli et al., 2004; Amadio et al., 2006). To confirm that in our model the MeSADP-mediated G-protein activation was mainly driven by P2Y1R, we also stimulated hippocampal membranes in the presence of the selective P2Y1R antagonist, MRS2179. MRS2179 was able to block the MeSADP-mediated response, confirming the P2Y1R involvement as no antagonistic effects have been demonstrated on P2Y12–13 receptors at the MRS2179 concentrations used (Moro et al., 1998; Von Kugelgen, 2006).

The results obtained on A1R–P2Y1R cross-talk in hippocampus showed that, stimulating one receptor, the functioning of the other was changed. In particular, P2Y1R pre-activation caused an impairment in A1R-G-protein coupling with a reduction in A1R agonist potency; on the other hand, A1R pre-activation induced an increase in P2Y1R functional coupling to G proteins. Our results are in agreement with the previously reported reduction in the A1R ligand affinities in cells expressing both A1R and P2Y1R (Yoshikoa et al., 2001). Various studies have reported that ATP, massively released after brain damage, acts to modulate not only its own P2YR but also A1R (Hourani et al., 1991; Piper & Hollingsworth, 1996; Masino et al., 2002; Fredholm et al., 2003; Yoshikoa & Nakata, 2004).

The functional consequence of this A1R–P2Y1R receptor cross-talk is complicated by the availability time and the balance of their endogenetic ligands. Extracellular ATP, rapidly available due to direct release into the extracellular space, and adenosine, available after ATP breakdown, are tightly regulated by rapid metabolism and re-uptake (Zimmermann, 2000) and can be differently regulated in physiological or pathological conditions; in fact the ecto-nucleotidase chain has proved to be up-regulated in ischemically damaged tissues (Braun et al., 1998). Data, at present, have shown the A1R–P2Y1R interaction mechanism may be used to fine-tune the purinergic signalling, including the inhibition of neurotransmission (Nakata et al., 2004). Considering the new information available and the A1R and P2Y1R involvement in glutamatergic transmission modulation (Mendoza-Fernandez et al., 2000; Masino et al., 2002; Kawamura et al., 2004; Rodrigues et al., 2005), we can speculate that there is an A1R–P2Y1R cross-talk in rat hippocampal glutamatergic synapses and surroundings glia, where these receptors are co-localized. This might therefore be one of the mechanisms for the adenine nucleotide-mediated inhibition of glutamatergic neurotransmitter release. Therefore, as suggested for adenosine A1 and A2a receptors in striatal (Ciruela et al., 2006) and hippocampal (Rebola et al., 2005) glutamatergic nerve terminals, a cross-talk/heteromerization of A1R–P2Y1R could exert a fine-tuning modulation of glutamatergic neurotransmission, providing a switch mechanism by which low and high concentrations of adenosine or purines could regulate glutamate release.

Because of the high level of complexity of purinergic receptor signalling (Volonté et al., 2006) and the regulation of glia–neuron and glia–glia communications by extracellular purines (Franke et al., 2006b; Jourdain et al., 2007), the present work opens the way to further investigation of the A1R–P2Y1R system interaction on astrocyte cell populations, which communicate bi-directionally with neurons (Newman, 2003; Bezzi et al., 2004; Jourdain et al., 2007) and contribute to damage or to regeneration after CNS injury (Franke et al., 2001; Anderson et al., 2003).

Acknowledgements
This work has been supported by a grant from the EU Marie Curie Fellowship Program [Marie Curie Training Site on Basic mechanisms of amino acid neurotransmission (BAMANI)], from the Italian Ministero dell’Università e della Ricerca Scientifica (MURST) (COFIN 2004 on ‘Purinergic receptors and neuroprotection’) and from the Research Council Norway (NFR).

Abbreviations
A1R, adenosine receptor A1; ADA, adenosine deaminase; CHA, N6-cyclohexyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GPCR, G protein coupled receptors; GTPγS, guanosine-5′-γ-thiotriphosphate; HSA, human serum albumin; HC, hippocampal membranes; MeSADP, 2-methylthio-adenosine 5′-diphosphate; MRS2179, 2′-deoxy-N5-methyl adenosine 3′,5′-diphosphate; P2Y1R, purinergic receptor P2Y1; PSD, postsynaptic density; TBST, Tris-buffered saline with Triton X-100. References
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Hippocampal A1–P2Y1 purine receptor cross-talk 901

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