

Cerebellar astrocytes co-express several ADP receptors. Presence of functional P2Y₁₃-like receptors

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Received 31 August 2004; accepted in revised form 21 October 2004

Key words: adenylate cyclase, ADP receptors, astrocytes, calcium, glial cells, nucleotide receptors, P2Y receptors

Abstract

Astrocytes exhibit a form of excitability based on variations of intracellular Ca²⁺ concentration in response to various stimuli, including ADP, ATP, UTP and dinucleotides. Here, we investigate the presence of the recently cloned ADP-sensitive receptors, P2Y₁₂ and P2Y₁₃ subtypes, which are negatively coupled to adenylate cyclase, in cerebellar astrocytes. We checked the effect of specific agonists, 2-methylthioadenosine diphosphate (2MeSADP) and ADP, on adenylate cyclase stimulation induced by isoproterenol. Both agonists significantly reduced the cAMP accumulation induced by isoproterenol. The inhibitory effect was concentration-dependent with IC₅₀ values of 46 ± 13 and 23 ± 14 nM for 2MeSADP and ADP, respectively. The experiments were carried out in the presence of MRS-2179, a specific antagonist of P2Y₁ receptor, to avoid any contribution of this receptor. Using fura-2 microfluorimetry we also proved that astrocytes responded to 2MeSADP stimulations with calcium responses in the absence and also in the presence of MRS-2179. Both effects, inhibition of adenylate cyclase and intracellular calcium mobilization, were not modified by 2MeSAMP, an antagonist of P2Y₁₂ receptor, suggesting that were mediated by P2Y₁₃-like receptors.

Abbreviations: 2MeSADP – 2-methylthioadenosine diphosphate; 2MeSAMP – 2-methylthioadenosine monophosphate; MRS-2179 – N6-methyl-2'-deoxyadenosine 3',5'-bisphosphate

Introduction

In recent years it has been demonstrated that astrocytes and other glial cells play a fundamental role in brain physiology by signalling to neurons and influencing their activity and *vice versa*. ATP and other nucleotides are involved in this neuron–glia interaction [1–3]. The extracellular actions of nucleotides are mediated by their interactions with specific membrane receptors, the P2-purinoceptors, subdivided into ionotropic P2X and metabotropic P2Y receptors [4]. Focusing on the metabotropic P2Y family, it comprises two structurally distinct subfamilies [5]. The first one is composed of P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ receptors, that exhibited different selectivity for adenine and uracile nucleotides, all exclusively coupled to phospholipase C pathway, except P2Y₁₁, which is also positively coupled to the cAMP pathway. The second subfamily includes P2Y₁₂, P2Y₁₃ receptors, both selective

for ADP, and the UDP-glucose receptor, renamed P2Y₁₄, which are all coupled to G_i. The identification of the P2Y₁₂ receptor has explained several ADP actions that were not mediated by P2Y₁ receptors [6–9]. One interesting example is the case of platelets, in which ADP-induced aggregation requires both P2Y₁ and P2Y₁₂ activation. In fact, the P2Y₁₂ receptor is the target of antithrombotic drugs, such as clopidrogel and ATP derivatives, the AR-C compounds, such as ARC69931MX [8, 10–13]. Another example is the G_i-coupled P2Y receptor distinct from P2Y₁, long known to exist in C6 glioma and B10 brain capillary endothelial cells, which has also been identified as P2Y₁₂ receptor [14–16]. Although to date there are not many data concerning the functionality of the P2Y₁₂ and P2Y₁₃ in neural cells, the high levels of expression found in brain suggests important roles in the nervous system [7, 17–20].

Our group has focused the investigation on the characterization of nucleotide receptors in cerebellum using purified cultures of neuronal and glial cells. We demonstrated that the two major neuronal types present in cerebellum, Purkinje cells and granule neurons, and astroglia expressed functional nucleotide receptors: Purkinje and granule neurons co-expressed ionotropic P2X and metabotropic P2Y nucleotide receptors and astrocytes

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only expressed functional P2Y nucleotide receptors [21–24]. Previous to the identification of the second subfamily of P2Y receptors the presence of $G_{i/o}$ -linked ADP receptors was detected in cerebellum, which was largely concentrated in the white matter [25, 26]. The cloning of the new members prompted us to search their presence in the glial cells. The current study reveals the presence of functional $G_{i/o}$ -linked ADP receptors in cerebellar astrocytes and provides molecular and biochemical evidences to suggest that these receptors are P2Y₁₃-like subtype.

Materials and methods

Cultures of rat cerebellar type-1 astrocytes

Type-1 astrocyte purified cultures were obtained from cerebellum of 7- to 8-day-old rats as previously described [27]. Cerebellar cells were dissociated by trypsin digestion and astrocytes purified by orbital shaken. They were grown until confluency in DMEM containing 10% (v/v) fetal calf serum, 30 mM glucose, 50 U/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml kanamycin and 2.5 µg/ml amphotericin at 37 °C in a humidified atmosphere of 5% CO₂/95% air. The type-1 astrocytes, finally, were passaged by trypsinization with 0.05% trypsin and 0.002% EDTA in phosphate-buffered saline without added Ca²⁺ and Mg²⁺. Cells were plated in 24-well Petri dishes for cAMP determinations or over 15-mm-diameter coverslips in 35-mm Petri dishes at a density of 200,000 cells/well for microfluorimetric and immunocytochemistry experiments.

Determinations of cAMP levels

Cells were washed for 2 h at 37 °C in 1 ml per well of Locke's solution (composition in mM: NaCl, 140; KCl, 4.5; CaCl₂, 2.5; KH₂PO₄, 1.2; MgSO₄, 1.2; glucose, 5.5; HEPES (acid), 10; pH 7.4) and incubated for 30 min in the presence of 500 µM rolipram. Then, cells were preincubated with 10 µM MRS-2179 for 3 min, previous to the stimulation with the agonists. Agonists were added for 3 min, and the incubations were terminated by addition of 6% trichloroacetic acid. Cell lysates were centrifuged at 13,000 g for 10 min at 4 °C and the cAMP levels in the supernatants were measured using the enzyme-immunoassay kit from Amersham.

Calcium imaging

Cells attached to coverslips were washed with Locke's solution supplemented with 1 mg/ml BSA and loaded with 5 µM fura-2/AM for 45 min at 37 °C. The coverslip was placed in a small superfusion chamber and the cells were imaged through a NIKON TE-200 microscope with a Plan Fluor 20×/0.5. Emitted light was isolated by a dichroic mirror (430 nm) and a 510-nm band pass filter (Omega Optical). The wavelength of the incoming light was selected with the aid of a monochromator (12-nm bandwidth, Perkin

Elmer Life Sciences) set at 340 and 380 nm. Twelve-bit images were obtained using an Ultrapix 2000 Mono CCD camera controlled by Ultraview PC software. Exposure time was 50 ms and change in the wavelength of incoming light from 340 to 380 nm is carried out in less than 5 ms. Time-course data represent the average light intensity in a small elliptical region within each cell. Background and autofluorescence components were subtracted at each wavelength and the F_{340}/F_{380} ratio was calculated. $[Ca^{2+}]_i$ was derived from F_{340}/F_{380} ratio using the equation's Grynkiewicz as previously described [21]. R_{max} and R_{min} parameters were obtained from an *in vitro* calibration performed by recording fluorescence from small droplets of fura-2 (free acid) dissolved in intracellular solution (in mM: 100 KCl, 10 NaCl, 1 MgCl₂, 10 MOPS and 1–2 µM fura-2, pH 7.0) plus 2.5 mM CaCl₂ (saturated Ca²⁺) or 2.5 mM EGTA (zero Ca²⁺). The dissociation constant of fura-2 was assumed to be 224 nM. Drugs were applied to the cells by superfusion in Locke's solution at 37 °C.

Immunocytochemical experiments

For immunofluorescence assay, coverslips were fixed with 4% PFA (w/v) for 15 min, washed twice in PBS and incubated for 1 h in PBS containing 3% BSA (w/v), 0.1% Triton X-100 (v/v), and 5% normal goat serum (v/v). Cells were washed twice with PBS/BSA and incubated with the primary antibodies for 1 h at 37 °C. Primary antibodies, diluted in PBS/BSA, recognized the specific rat proteins: mouse anti-glial fibrillar acid protein (GFAP, 1:200, Sigma), rabbit anti-P2Y₁ (1:100, Chemicon) and anti-P2Y₁₂ (1:50, Alomone). Cells were then washed three times and incubated for 1 h at 37 °C with the appropriated secondary antibodies: Goat anti-mouse IgG fluorescein conjugated (1:1,000, Sigma) and goat anti-rabbit IgG rhodamine conjugated (1:500, Sigma). After this incubation, cells were washed three times and mounted following standard procedures with Prolong antifade medium (Molecular Probes). Controls of unspecific labeling were made in the same experimental conditions but the primary antibodies were replaced with the same volume of PBS/BSA solution. Coverslips were viewed with a NIKON microscope equipped with a 60× 1.4 oil objective and fluorescein/rhodamine filters.

RT-PCR experiments

Total RNA was isolated from astrocyte cultures from one passage and plated onto 35-mm-diameter culture dishes, using the RNeasy system (RNeasy extraction kit, Qiagen). Extracted RNA was routinely submitted to digestion with RNase-free DNase to eliminate traces of contaminating genomic DNA (DNA extraction kit, Qiagen), which could interfere in the PCR experiments. RNA was accurately measured using the sensitive fluorescent probe Ribogreen (RNA Quantitation Reagent, Molecular Probes).

cDNA was synthesized from 1 µg total RNA using the MultiScribe Reverse Transcriptase (1.25 U/ml, Applied

Biosystems), according to manufacturer instructions. Parallel RT reactions for each RNA sample were carried out in the absence of the reverse transcriptase enzyme, to evaluate the degree of genomic DNA contamination (-RT control). For PCR reactions, 2–5 µl of the RT reaction product were amplified at 60 °C annealing using 0.625 U of Ampli Taq Gold DNA polymerase (Applied Biosystems) and specific oligonucleotide primers for P2Y receptors. The following forward (Fw) and reverse (Rv) oligonucleotide primers obtained from rat cDNA sequences were used:

P2Y ₁	Fw : 5'–CATTCTTTATTTCTGGCTGGAGAT–3'
	Rw : 5'–GAGTCATTTCTTCACTCTGGATTGTA–3'
P2Y ₁₂	Fw : 5'–CAGGTTCTTCCCATTTGCT–3'
	Rw : 5'–CAGCAATGATGATGAAAACC–3'
P2Y ₁₃	Fw : 5'–TACCTCAAGAACTCTGGTGGCAGAC–3'
	Rw : 5'–TTCACGGATGATGGCGTTGCCTCTTGTT–3'
P2Y ₁₄	Fw : 5'–TGTCTGCCGTGATCTTCT–3'
	Rw : 5'–GGGTCCAGACACACATTG–3'

Parallel PCR reactions were carried out with RT products obtained from platelets, spleen and hippocampus as positive expression controls for P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors, respectively.

Statistical analysis

Results are represented as the means ± SE calculated from at least six experiments performed in duplicate from different cultures. Comparisons were made by one-way ANOVA, and Dunnett post-test analysis was only applied when a significant ($P < 0.05$) main effect was indicated by the ANOVA.

Results

To analyze the presence of G_i-linked ADP receptors in cerebellar astrocytes, we used several strategies, RT-PCR analysis for the detection of the mRNA codifying for P2Y₁₂ and P2Y₁₃ receptors, immunocytochemical studies

to identify the corresponding proteins and biochemical and pharmacological approaches to prove their functionality.

Expression of mRNA for P2Y_{1,12,13,14} receptors in cerebellar astrocyte cultures

RT-PCR analysis showed that cerebellar astrocytes co-expressed several P2Y receptors of the ADP receptor family. Besides the P2Y₁ receptor, whose functionality in astrocytes has been previously demonstrated, P2Y₁₂ and P2Y₁₃ mRNA were abundantly expressed in astrocytes in a similar extent to that found in platelets and spleen, respectively, in the same experimental conditions (Figure 1). The expression of the other member of the subfamily, the P2Y₁₄ receptor, was also detected. In all cases the specific bands of the PCR products were of the expected sizes and no signals were observed in control assays in the absence of templates.

Inhibition of cAMP production by G_i-linked ADP receptors

Based on the fact that cerebellar astrocytes possess β-adrenergic receptors coupled to adenylate cyclase activation, the presence and functionality of G_i-linked ADP receptors was investigated by analyzing the effect of specific agonists on cAMP production induced by isoproterenol. All these experiments were carried out in the presence of a specific antagonist of the P2Y₁ receptor, MRS-2179, to exclude any action of the agonists through this receptor. Adenosine deaminase was also added in the incubations to avoid any effect of the nucleoside produced by extracellular hydrolysis of nucleotides. In previous work we demonstrated that astrocytes possessed A_{2B} adenosine receptors coupled to adenylate cyclase stimulation [27]. Figure 2 shows that co-stimulation of astrocytes with 2MeSADP or ADP and isoproterenol significantly reduced the isoproterenol-increased cAMP levels in a dose-dependent manner, reaching a maximum of 40% reduction at 10 µM concentration. The IC₅₀ values were 46 ± 13 and 23 ± 14 nM for 2MeSADP and ADP, respectively. The similar potency displayed by these agonists suggests that the ADP receptor that mediates this inhibition was a P2Y₁₃-like

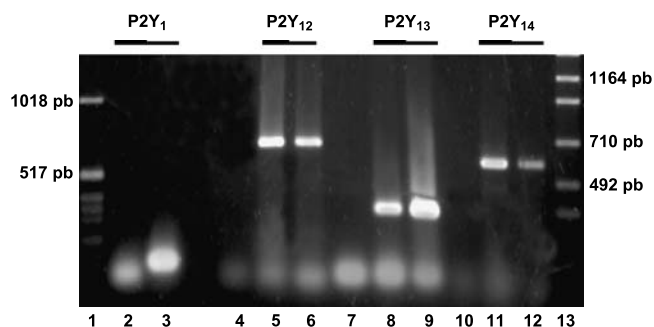


Figure 1. Expression of mRNAs for P2Y₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄ nucleotide receptors in cerebellar astrocyte cultures. Typical RT-PCR results obtained from three different cultures. PCR products for P2Y₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors in astrocytes are shown in lanes 3, 5, 8 and 11, respectively. Expression controls were made using total RNA obtained from platelets for P2Y₁₂ (lane 6), spleen for P2Y₁₃ (lane 9) and hippocampus for P2Y₁₄ receptors (lane 12). Besides the H₂O controls performed in the absence of template are shown in lanes 2, 4, 7 and 10. Band sizes were determined using RNA standards (lanes 1 and 13).

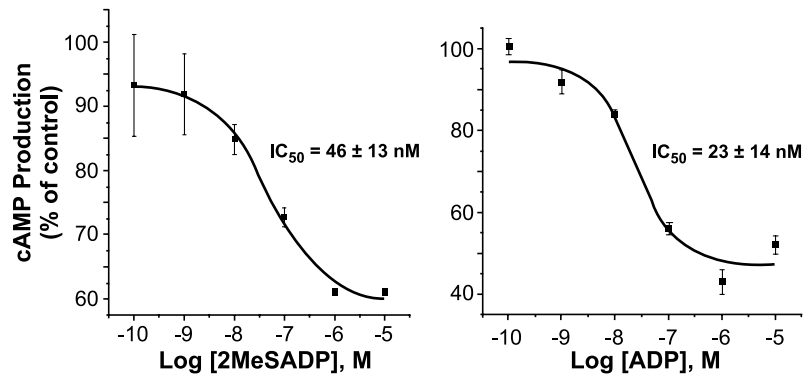


Figure 2. Identification of ADP receptors that inhibit isoproterenol-activated adenylate cyclase in cerebellar astrocytes. Astrocytes were preincubated with 10 μ M MRS-2179 previous to co-stimulation for 3 min with different concentrations of purinergic agonists (2MeSADP or ADP) and 10 μ M isoproterenol, and the cAMP accumulation was calculated as described in Materials and methods. Data were normalized with respect to the control for each experiment. Values are the means \pm SD of five experiments performed in triplicate. The logistic curves were fitted by non-linear regression.

receptor. To validate the involvement of the 'putative' P2Y₁₃ receptors we examined the action of a P2Y₁₂ receptor antagonist, 2MeSAMP [8, 28]. Since both agonists exhibited the same potency, and ADP can be hydrolyzed more quickly than 2MeSADP, 2MeSADP was used in the following experiments. Preincubation of astrocytes with the nucleotide derivative, 2-methylthioadenosine 5'-monophosphate (2MeSAMP, 100 μ M), did not revert the inhibition displayed by 2MeSADP (Figure 3). The diagram also shows that simple stimulation of astrocytes with 10 μ M 2MeSADP or 100 μ M 2MeSAMP did not change basal cAMP levels. In addition, 2MeSAMP had no effect on cAMP accumulation stimulated by isoproterenol. Finally, it was tested whether the inhibition of adenylate cyclase by P2Y agonists was associated with a G_{oi} protein. For this purpose astrocytes were preincubated overnight with 100 ng/ml pertussis toxin previous to carry out cAMP determinations. This pretreatment completely abolished the inhibitory effect of 2MeSADP, indicating the involvement of a G protein of the PTX-sensitive G_i/G_o family (Figure 3).

2MeSADP-induced calcium responses in cerebellar astrocytes

Given that P2Y₁₃ receptor was also coupled to phospholipase C activation and the subsequent inositol phosphate production [7], we investigated its possible running in cerebellar astrocytes. Cells were challenged with 10 μ M 2MeSADP and single cell responses followed by microfluorimetry. As expected cells responded to 2MeSADP with rapid [Ca²⁺]_i transients (Figure 4). The $\Delta F_{340}/F_{380}$ were highly variable from cell to cell ranging from 0.022 to 0.088 (0.051 ± 0.002 , $n = 224$ cells, corresponding to [Ca²⁺]_i increases of 360 ± 45 nM). But, surprisingly most of tested cells also responded to 2MeSADP stimulations in the presence of the specific P2Y₁ antagonist, MRS-2179, which reveals that other ADP-sensitive receptor was being activated. Although the magnitude of the calcium responses observed in the presence of MRS-2179 was very heterogeneous. We could distinguish three groups of cells: Cells that were nearly insensitive to MRS-2179 (38% of

cell population, population A), cells partially sensitive to MRS-2179 antagonism, which showed between 31% and 70% of control cell responses (49% of tested cells, population B), and cells in which 2MeSADP responses were completely abolished by MRS-2179 (13% of tested cells, population C). The same distribution pattern of calcium responses was obtained after preincubation with the two antagonists MRS-2179 and 2MeSAMP.

Finally, we also carried out immunocytochemistry experiments to detect the presence of P2Y₁₂ protein. Figure 5 shows that there was no specific labeling of astrocytes with the anti-P2Y₁₂ receptor antibody, whereas, as expected, a specific staining was observed with the anti-P2Y₁ receptor antibody in the same experimental conditions. Positive controls for the P2Y₁₂ receptor detection were made in platelets (results not shown).

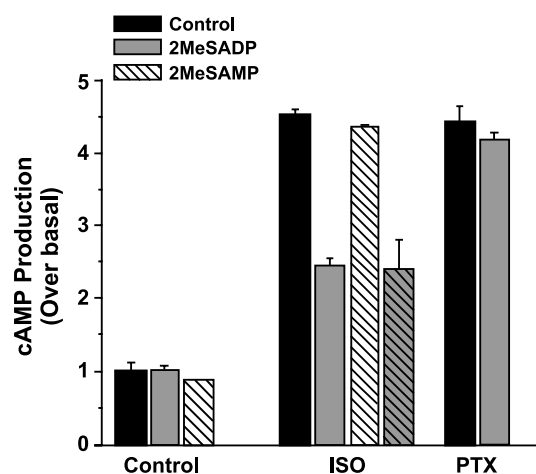


Figure 3. Effect of 2MeSAMP and pertussis toxin on the 2MeSADP-induced inhibition of cAMP production stimulated by isoproterenol in astrocytes. To check the effect of 2MeSAMP, cells were preincubated with 10 μ M MRS-2179 and 100 μ M 2MeSAMP for 3 min previous to co-stimulation with 10 μ M 2MeSADP and isoproterenol and cAMP production measured as described in Materials and methods. Where indicated, cells were stimulated with 10 μ M 2MeSADP or 100 μ M 2MeSAMP alone or in the presence of isoproterenol for 3 min. To study the effect of pertussis toxin cells were treated overnight with 100 ng/ml pertussis toxin. Data were normalized with respect to the controls for each experiment. Values are the means \pm SD of four experiments performed in quadruplicate from different cultures.

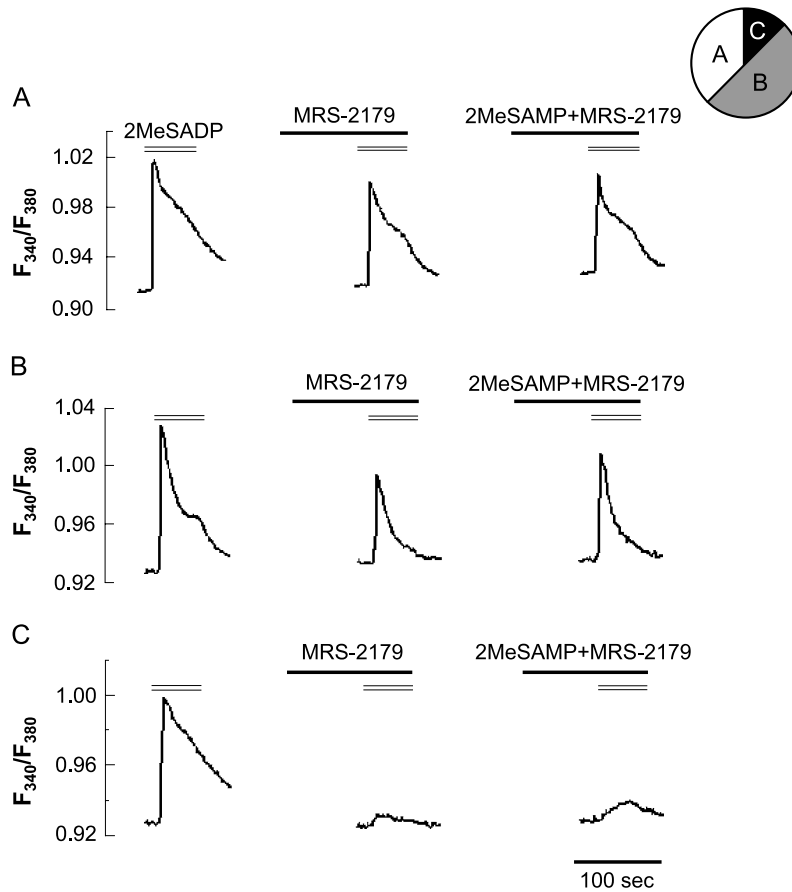


Figure 4. Calcium responses elicited by 2MeSADP stimulation in single cerebellar astrocytes. Cells were loaded with fura-2 and the time-course of fluorescence changes recorded from cells after stimulation with 10 μ M of the agonist for 40 s as described in Materials and methods. Where indicated, before cells were stimulated with 2MeSADP, they were preincubated for 3 min with 10 μ M MRS-2179 in the absence (middle recordings) or presence of 100 μ M 2MeSAMP (right recordings). Figure shows the calcium responses of three cells, which are representative of the cell subpopulations found with respect to their sensitivity to MRS-2179 antagonism (represented in the diagram, $n = 300$ cells tested from three different cultures).

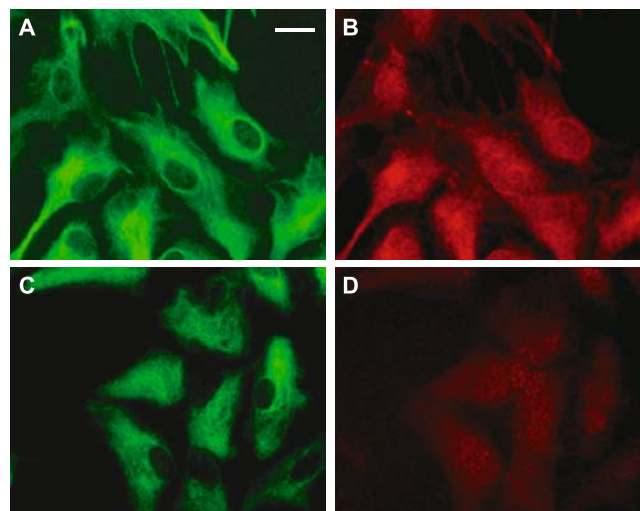


Figure 5. Fluorescence images of cerebellar astrocytes labelled with different antibodies. Panels A and C show specific anti-GFAP-FITC immunostaining of the cells. (B) Same field as A viewed with Rhodamine optics using anti-P2Y₁-TRITC reveals specific labelling of the cells. (D) Same field as C viewed with Rhodamine optics using anti-P2Y₁₂-TRITC shows a negative immunolabelling. These images show the purity of the cultures, all the cells are identified as astrocytes. Bar = 20 μ m.

Discussion

In the present study we show the presence of functional G_i -linked receptors in cultured astrocytes from rat cerebellum. The pharmacological profile of the analyzed responses to 2MeSADP and ADP suggests the involvement of P2Y₁₃-like receptor subtype, which represents the first report of a functional P2Y₁₃-like receptor expressed in native cellular environment. The identification and characterization of G_i -linked receptors, such as P2Y₁₂ and/or P2Y₁₃ in native tissues is quite difficult, particularly when they could be co-expressed with other P2Y subtypes, such as P2Y₁. These three subtypes are ADP receptors with similar agonist profile (2MeSADP \geq 2MeSATP $>$ ADP \geq ATP) and, in addition, there are no specific antagonists for each one of the subtypes. Whereas P2Y₁ receptor can be selectively blocked by MRS-2179, the AR-C compounds described as specific antagonists of P2Y₁₂ receptor can also act on some P2Y₁₃ [8, 9, 29–32]. The pharmacological studies reported in the literature with the cloned P2Y₁₂ and P2Y₁₃ receptors revealed that they could be distinguished by the relative potency of 2MeSADP and ADP. At the platelet P2Y₁₂ and brain P2Y₁₂ like receptor 2MeSADP is more potent than ADP (two or three orders of magnitude) and clearly differs from the P2Y₁₃ at which both agonists are nearly equipotent [8, 28, 30, 31]. In cerebellar astrocytes 2MeSADP and ADP exhibited similar potency in decreasing cAMP accumulation induced by isoproterenol. Although it is not accurate to compare the relative potency of agonists described for the different cloned receptors, the IC₅₀ values reported here were very similar to those described for the human and mouse P2Y₁₃ receptors (IC₅₀ = 13 nM). The values are critically dependent on the experimental conditions, such as expression levels of receptor in the different cell lines and effector system assessed, cAMP and/or inositol phosphate production, fluorimetric imaging plate reader assays, binding of radiolabelled agonists, etc. The recent cloning and pharmacological characterization of P2Y₁₃ receptor

from rat tissues has revealed that ADP is two to three times more potent than 2MeSADP on this receptor, which agrees with our results [32].

Others findings that supported the involvement of a P2Y₁₃-like receptor are that 2MeSADP effects, both the repression of cAMP levels and the calcium responses recorded in the presence of MRS-2179, were not reversed by 2MeSAMP, which is a selective antagonist of P2Y₁₂ receptor. 2MeSAMP (10 and 50 μ M) completely abolished the ADP and 2MeSADP effects mediated by the P2Y₁₂ receptor [8]. In addition, we have not detected the presence of P2Y₁₂ protein by immunocytochemistry using a specific antibody for the hP2Y₁₂, whereas the presence of P2Y₁ protein was confirmed in the same experimental conditions. These negative results supported the involvement of another G_i -linked ADP receptor. Although we cannot exclude the possibility that P2Y₁₂ receptor could be co-expressed in some cells in a very low quantity below detection limit in our experimental conditions. It is interesting to take into account that the detection of mRNA does not always correlate with the presence of the corresponding protein. In this sense, we have also found by RT-PCR bands that corresponded to mRNA for some P2X subunits (P2X₁, P2X₃ and P2X₄) in our astrocyte cultures (results not shown) but we have not detected any ionotropic nucleotide responses. In contrast to these findings, the presence of ionotropic P2X receptors in astrocytes from other brain areas has been demonstrated [33–35]. This heterogeneity might be related to the need to regulate the different neurotransmission types.

To conclude, the present study reveals that cerebellar astrocytes possess at least two specific ADP receptors, which can be simultaneously activated at nanomolar concentrations of the nucleotide, P2Y₁ and P2Y₁₃-like receptors, coupled to phospholipase C stimulation and adenylate cyclase inhibition, respectively (Figure 6). The question that arises is: What is the function of these receptors in astrocytes? As previously mentioned, it is clearly demonstrated that P2Y₁ and P2Y₁₂ cooperate to induce

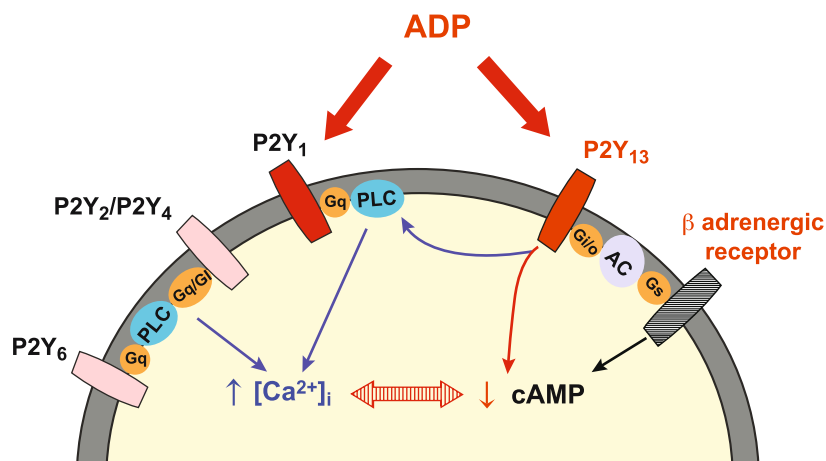


Figure 6. Schematic representation of P2Y nucleotide receptors present in cerebellar astrocytes. Astrocytes coexpressed several P2Y receptors that can be selectively stimulated by adenine and uracile nucleotides. ADP would interact with P2Y₁ and P2Y₁₃-like receptors triggering two intracellular signaling cascades, intracellular calcium mobilization and decreasing cAMP production.

ADP-induced platelet aggregation, a similar cooperation between P2Y₁ and P2Y₁₃-like receptor could be take place in astrocytes to control metabolism, proliferation, differentiation or even apoptosis depending on cellular environment or otherwise function independently. The fact that P2Y₁₃-like receptor stimulation also induced calcium responses might suggest that P2Y₁₃ would substitute P2Y₁ receptor, when the later was in inactive state.

Acknowledgements

This work was supported by grants from Ministerio de Ciencia y Tecnología (BFI2002-03626) and Comunidad Autónoma de Madrid (CAM 88.5/0004.1/2003). L.M.G. Carrasquero is a fellow from Ministerio de Educación.

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