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Immunolocalisation of P2Y receptors in the rat eye

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Abstract

Nucleotides present an important role in ocular physiology, which has been demonstrated by recent works that indicate their involvement in many ocular processes. P2Y are important among P2 receptors since they can control tear production, corneal wound healing, aqueous humour dynamics and retinal physiology. Commercial antibodies have allowed us to investigate the distribution of P2Y receptors in the cornea, anterior and posterior chamber of the eye and retina. The $P2Y_1$ receptor was present mainly in cornea, ciliary processes, and trabecular meshwork. The $P2Y_2$ receptors were present in cornea, ciliary processes and retinal pigmented epithelium. $P2Y_4$ was present in cornea, ciliary processes, photoreceptors, outer plexiform layer and ganglion cell layer. The $P2Y_6$ presented almost an identical distribution as the $P2Y_4$ receptor. The $P2Y_{11}$ was also detectable in the retinal pigmented epithelium. The detailed distribution of the receptors clearly supports the recent findings indicating the relevant role of nucleotides in the ocular function.

Introduction

There is a general knowledge of nucleotides acting as extracellular messengers in tissues [1]. Among the tissues/organs which are under investigation, the eye is one that has not been fully investigated; it has been taken into consideration only in recent times, mainly because nucleotides seem to have interesting physiological roles and putative therapeutic applications (for a review, see Pintor [2]).

A quick review of the existing literature in the nucleotide field of the eye will emphasise the importance of metabotropic P2Y receptors in the ocular structure. For instance, P2Y receptors can produce an increase in the proportion of the mucin layer in the tear film [3]. Also, uridine nucleotides can modify chloride currents facilitating the production of the aqueous component of the tear [4, 5]. Inside the eye, P2Y receptors are able to regulate the production and the drainage of the aqueous humour due to their presence in the ciliary processes and trabecular meshwork cells [6, 7]. Finally, metabotropic nucleotide receptors have been described in the neural and non-neural retina [8–11].

Despite the fact that P2Y receptors seem widely distributed in the ocular surface and in other ocular areas, one needs to be aware that frequently the existence of those receptors has been pharmacologically demonstrated in primary cell cultures or in cell lines rather than in the native tissues. Apart from this, another important point is that the cells under investigation may contain more than one P2 purinoceptor subtype. This fact makes difficult the interpretation of the pharmacological data, avoiding very often a clear picture of the P2 receptors present in a tissue. For these reasons, in the present experimental work we present the distribution of P2Y receptors in the rat eye by means of commercial antibodies. We hope that this 'picture' will help researchers to better understand the role of nucleotides in the eye.

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Materials and methods

Immunohistochemistry

A total of 10 Wistar rats of 13 days postnatal (P13) were sacrificed by rapid decapitation. For the immunohistochemical study, the eyes were removed and were fixed overnight at 4 °C, using 4% paraformaldehyde in phosphate buffer, pH 7.2. After fixation, the eyes were submitted to a cryoprotective process. Sections of 14 μm were made using a Leica 3050 M cryostate. Immunohistochemical studies were performed starting with the following primary antibody dilutions: anti-$P2Y_1$, 1/200; anti-$P2Y_2$, l/500; anti-$P2Y_4$, 1/500; anti-$P2Y_6$, 1/200 and

anti-$P2Y_{11}$, l/1,000. As secondary antibody we used goat anti-rabbit IgG-TRITC from Sigma (T-6778). In the case of double immunostaining, we used as primary antibodies mouse anti-synaptophysin (Sigma, S-5768) 1/250, as a neuronal marker, mouse anti-vimentin (Sigma, V-6630) 1/500, as a marker of the protein vimentin. As secondary antibodies we used goat anti-IgG mouse-FITC (Sigma, F-4014), 1/500 in the case of anti-synaptophysin marker, and goat anti-IgM mouse-FITC (Sigma, F-9259), l/100 for the anti-vimentin marker. Controls were carried out by following the same procedures but the primary antibody was substituted by the same volume of PSS/BSA solution.

Eye sections were analysed by confocal microscopy using a Zeiss Axiovert 200M microscope equipped with a LSM5 Pascal confocal module. Sections were observed with a Zeiss 63× oil immersion lens, numerical aperture 1.40. FITC was monitored by excitation with the 488-nm wavelength laser, and TRITC was excited at 543-nm wavelength. All the images were managed with the LSM5 Pascal software.

Western blotting

For Western blot analysis, the eyes were rapidly removed and the different parts were placed on ice and subsequently homogenised with lysis buffer that contains HEPES 50 mM pH 7.5, Triton 2.5% (w/v), EDTA 10 mM, PMSF 0.2 mM and leupeptin 5 μg/ml. Protein samples (40 μg) were separated by SDS-PAGE (10% acrylamide gel) using the Bio-Rad Mini-Protein® 3-Cell System. Proteins were transferred to nitrocellulose membranes. Following transfer, the membranes were washed, blocked and incubated. The dilutions of primary antibodies were as follows: anti-$P2Y_1$, 1/200; anti-$P2Y_2$, 1/500; anti-$P2Y_4$, l/200; anti-$P2Y_6$, l/200 and anti-$P2Y_{11}$, l/1000. As secondary antibody mouse anti-rabbit IgG coupled to horseradish peroxidase, from Sigma (A-2074) at 1/1000 dilution were used. Blots were developed using the Enhanced Chemiluminiscence detection system (Amersham).

Chemicals

Antibodies against the P2Y receptors were purchased from Alomone Labs (Israel), except $P2Y_{11}$ which was a gift from Dr D. Cousens (Glaxo-Smithkline). Anti-synaptophysin and anti-vimentin antibodies were from Sigma (St. Louis, Missouri, USA). Other reagents were analytical grade from Merck (Darmstadt, Germany).

Results

P2Y receptors in the cornea

The cornea is the most external and transparent part of the eye. This eye region is formed by five to six different layers. The most relevant ones include the epithelium, the most superficial one; the stroma, the thickest; and endothelium the inner one. The distribution of the purinergic receptors present in the cornea can be carried out by making slices and performing immunohistochemistry, as described in Materials and methods. The analysis of the P2Y receptors present in corneal slices, by means of the available P2Y receptor antibodies, showed that $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ antibodies labelled this part of the eye. Among the different areas of the cornea, the one which offered positive staining for all receptors was the epithelial layer (Figure 1), although the intensity of the labelling was not the same for all the tested antibodies and for the different areas examined (see Table 1). For example, the stroma showed a minor label in all the cases (it only contains some keratinocytes) while the endothelium (the most inner part of the cornea) gave positive results with the $P2Y_2$, $P2Y_4$ and $P2Y_6$ antibodies (Table 1).

P2Y receptors in the iris and ciliary body

The presence of $P2Y_1$, $P2Y_2$ and $P2Y_4$ was demonstrated (Table 1) in the iris, which is the structure that works as a diaphragm, modifying the amount of light entering the eye. In particular, one of the better staining was achieved with the $P2Y_4$ receptor antibody (Figure 2). Concerning the structure of the iris, formed by the dilator muscle and the sphincter, we tried to see whether or not there were differences between these two parts concerning the $P2Y_4$ receptor. We have concluded that there were no differences between these two regions of the iris.

The ciliary body (ciliary processes) is the part of the eye where the aqueous humour is synthesised. The physiology of this area is relevant since it is one of the ocular structures contributing to keep the right pressure within the eye. An augment of this intraocular pressure can trigger a pathology termed glaucoma.

The ciliary body contains two types of epithelial cells, non-pigmented and pigmented ciliary epithelial cells. The non-pigmented cells are facing the posterior chamber where the release of the aqueous humour occurs. The pigmented epithelial cells are just underneath the non-pigmented and they limit the stroma that contains a fenestrated endothelium, which supplies this ocular region. With the intermediate filament antibody vimentin, it is possible to visualise the non-pigmented epithelium and lens capsule (see Figure 3). This antibody helps to identify these two regions and provides interesting information when it is applied together with the P2Y receptor antibodies.

The studies performed with the P2Y receptor antibodies demonstrated that $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ gave positive labelling in this region (Figure 3). Nevertheless, the distribution was not the same for each antibody. $P2Y_1$, $P2Y_2$ and $P2Y_4$ marked both, non-pigmented and pigmented epithelial cells. In these cases, it was possible to see that the stroma was also labelled. A different picture was obtained with the $P2Y_6$ receptor antibody. $P2Y_6$ antibody is concentrated exclusively in the stromal area and is not a positive labelling in the epithelia (Figure 3).

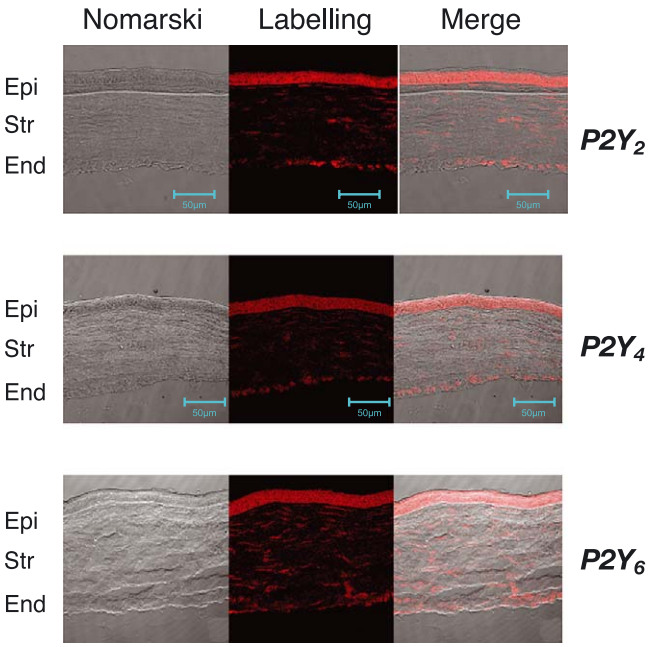

Figure 1. Presence of P2Y receptors in the cornea. Corneal sections labelled as indicated in Materials and methods, presented positive staining to $P2Y_2$ (upper panel), $P2Y_4$ (mid panel) and $P2Y_6$ (lower panel), in the epithelium, and in some cases in the endothelium.

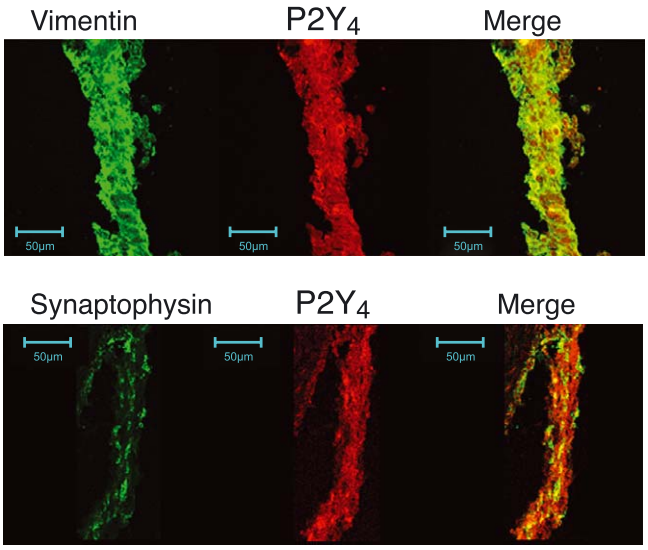

Figure 2. Presence of $P2Y_4$ receptors in the iris. Iris sections presented positive staining to $P2Y_4$ receptor antibody (in red). Vimentin (to label intermediate filaments) or synaptophysin (nerve terminals) in green demonstrated some co-localisation between $P2Y_4$ receptors and the other markers.

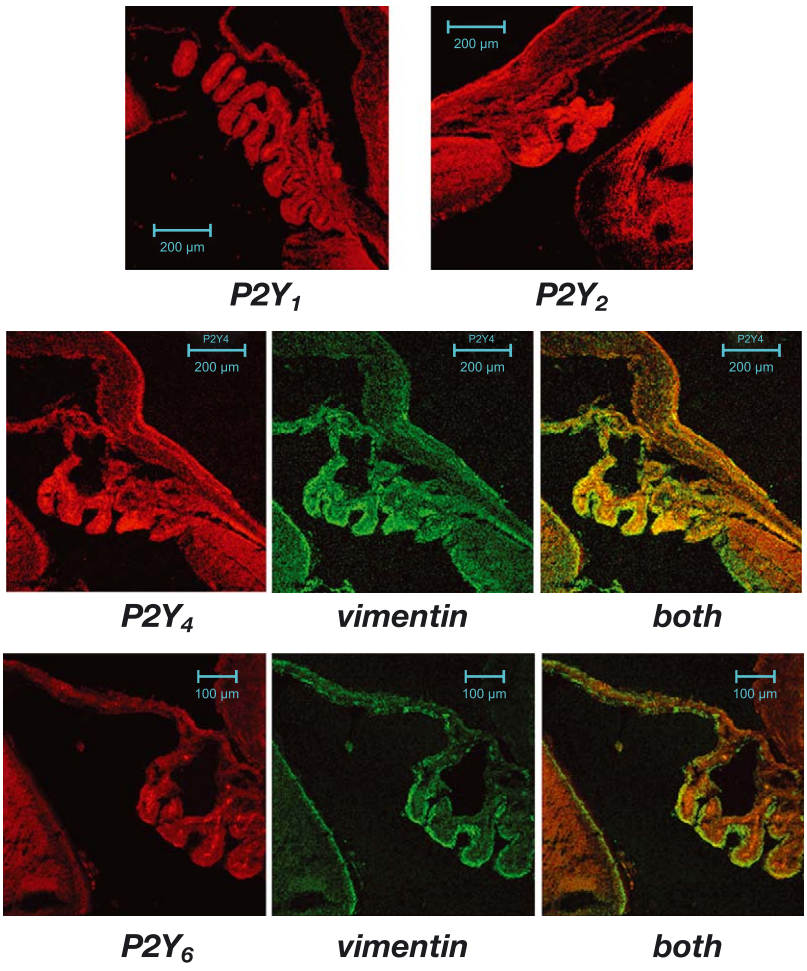

Figure 3. P2Y receptors in the ciliary body and related areas. Positive labelling to $P2Y_1$, $P2Y_2$ (upper panel), $P2Y_4$ (mid panel) and $P2Y_6$ (lower panel) were observed in the ciliary body (in red). It is noteworthy that the distribution of the receptor depends on the particular P2Y subtype. $P2Y_2$ labelled all the ciliary body while $P2Y_6$ mainly labelled the stromal area. Vimentin (in green) labels the non-pigmented epithelial cells as well as the lens capsule and helps to understand the distribution of some of the P2Y receptors.

Table 1. Distribution of P2Y receptors in the anterior segment of the eye.

Receptor	Corneal epithelium	Corneal endothelium	Pigmented epithelial cells	Non-pigmented epithelial cells	Trabecular meshwork	Iris
$P2Y_1$	++	+	+	++	++	+
$P2Y_2$	+++	+	++	++	++	+
$P2Y_4$	++	+	+	++	−	++
$P2Y_6$	++	+	−	−	−	−
$P2Y_{11}$	−	−	−	+	−	−

−, No labelling, +, low labeling, ++, moderate labelling, +++, strong labelling.

Another important area close to the ciliary processes is the trabecular meshwork. This area is responsible for the drainage of the aqueous humour, thereby regulating intraocular pressure. The immunohistochemical study of this area permitted us to detect the presence of $P2Y_1$ and $P2Y_2$ receptors, but it was not possible to visualise any of the other tested (Table 1).

P2Y receptors in the retina

The retina has also been investigated for the presence of P2Y receptors. Sections of the retina were incubated with P2Y receptor antibodies as well as with vimentin, which behaves as a glial cell marker (Müller cells marker) in this region, and synaptophysin, which is a neural marker.

Taking together the combination P2Y/vimentin and P2Y/synaptophysin allowed us to not only see the presence of the purinoceptors, but also made it possible for us to allocate them with specific areas of the retina.

Three P2Y receptor antibodies labelled the retina, the $P2Y_2$, $P2Y_4$, and $P2Y_6$, (Table 2). $P2Y_2$ presented a strong labelling in the non-neural retina, i.e. in the retinal pigmented epithelium (RPE), while other areas were not significantly stained (Figure 4). It was also possible to observe inmunolabelling against the $P2Y_{11}$ antibody on the RPE, this being the only area where this receptor seems to be present (Figure 4). The RPE was also labelled with the $P2Y_4$ antibody, although other areas of the neural retina were also positive. In this sense, the outer segments of the photoreceptors, the outer plexifom layer and the ganglion cell layer, were stained (Figure 5). The $P2Y_6$ presented a similar distribution pattern labelling the outer segments of the photoreceptors, outer plexiform layer and ganglion cell layer. Moreover, the inner plexiform layer was also labelled with the $P2Y_6$ receptor antibody in clear contrast with the $P2Y_4$ (Table 2).

Studies on the presence of P2Y receptors in Müller cells were performed with vimentin. These studies demonstrated the presence of $P2Y_4$ and $P2Y_6$ receptors in this glial cells (Figure 6). It was not possible to observe any co-localisation (results not shown) when the other P2Y receptors were assayed together with the vimentin.

Western blot analysis

Western blots were carried out by taking samples of the different areas under study following the protocol described in Materials and methods. Using this technique, we were able to confirm the existence of those P2Y receptors previously shown in the immunohistochemical studies. It is important to point out that we had some problems in the retinal preparations to get a positive band against the $P2Y_6$ receptor. We still do not know the reason why it is not possible to obtain a positive Western blot for this receptor. In contrast, it was possible to see the mentioned band in the corneal extracts (Figure 7). $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors presented molecular weights of 42, 50, 92 and 97 kDa, respectively. The other antibodies, like the one for the $P2Y_{11}$, did not reveal any positive band.

Discussion

P2Y receptors in the cornea and sclera

Metabotropic receptors for nucleotides in the cornea have been identified by means of in situ hybridisation techniques. $P2Y_2$ receptors are predominantly expressed in the cornea, sclera, goblet cells and meibomian glands of monkeys and rabbits [12]. In our experiments, we describe $P2Y_2$ receptors and the presence of $P2Y_1$, $P2Y_4$ and $P2Y_6$. The differences between our results and those described in the

Table 2. Distribution of P2Y receptors in the retina.

Receptor	Reinal pigmented epithelium	Photoreceptors	Inner plexiform layer	Outer plexiform layer	Ganglion cell layer
$P2Y_1$	−	−	−	−	−
$P2Y_2$	+++	+	−	−	−
$P2Y_4$	+	+++	−	++	++
$P2Y_6$	−	+++	+	++	++
$P2Y_{11}$	−	−	−	−	−

−, No labelling, +, low labeling, ++, moderate labelling, +++, strong labelling.

literature can be due to the animal model used. In the rabbit corneal epithelium the possible existence of $P2Y_4$ and $P2Y_6$ receptors cannot be discarded. Experiments studying the effect of nucleotides on corneal wound healing demonstrate that, apart from the involvement of a $P2Y_2$ receptor in the re-epithelialisation process, the participation of $P2Y_4$/$P2Y_6$ receptors needs to be taken into consideration [13].

In the endothelium, the existence of receptors for ATP has been demonstrated. These receptors are of the P2Y subtype, although there are not a detailed description of the particular subtype [14]. We may suggest, according to our results, that $P2Y_2$ and $P2Y_4$ are good candidates to fulfill the results described by Srinivas, who clearly identified different responses, therefore suggesting the presence of more than one P2Y receptor subtype.

$P2Y_4$ receptors have been also identified by in situ hybridisation techniques in the conjunctiva, and a role on the CI^- movement on conjunctival epithelial cells has been suggested. Hosoya et al. [15] indicate that $P2Y_2$ and $P2Y_4$ receptors control the ion flux across the conjunctiva. This ion flux can be measured by activating $P2Y_2$ receptors with UTP. This receptor stimulation produces a clear movement of CI^- ions and the concomitant fluid transport [16].

Conjunctival goblet cells can modulate the release of mucins by means of $P2Y_2$ receptors, as suggested by Dartt [17] and Murakami and co-workers [l8].

P2Y receptors in the iris ciliary body

There is not much information about the presence of P2 receptors in the iris. We have observed the presence of $P2Y_1$, $P2Y_2$ and mainly $P2Y_4$. Previous functional studies demonstrated the presence of P2Y receptors, although nothing indicated the receptor subtype. In these studies, Fuder and co-workers [19] demonstrated the effect of ATP on the iris, and how this effect is mediated by a P2Y-like receptor [20]. P2Y receptors present in the iris may regulate the pupil size by either controlling the muscle contraction/relaxation or by modulating the sympathetic nervous system which controls the iris physiology [20].

Concerning the ciliary body, the presence of P2Y receptors has been demonstrated in pigmented and non-pigmented epithelial cells by measuring IP3 generation and cytosolic Ca^{2+} levels [21]. Other experiments performed by Shaindullah and Wilson [22] also demonstrate that the effects of UTP and ATP are equipotent and that the receptors they activate may belong to the $P2Y_2$ purinoceptor subtype. In our case, we have been able to identify different P2Y receptors; nevertheless, we were able to see a differential distribution of the receptors in the ciliary processes. $P2Y_1$, $P2Y_2$ and $P2Y_4$ showed immunoreactivity in the ciliary non-pigmented and pigmented epithelium. The existence of $P2Y_2$ receptors in non-pigmented and pigmented epithelial cells has been described by in situ hybridisation techniques [12]. This $P2Y_2$ receptors and also $P2Y_1$ have been described in the rabbit ciliary body epithelium by means of functional studies that reveal the metabotropic nature of these receptors [7]. These receptors, when activated, can increase cytosolic Ca^{2+} levels plus PGE_2 and cAMP. This combination produces the activation of chloride channels which reduce the aqueous humour formation and a reduction in IOP [23]. The $P2Y_6$ receptor appears only in the stroma of the ciliary processes. This fact could be due to the existence of a fenestrated blood vessel network in this area. Since $P2Y_6$ receptors have been described in the endothelium of blood vessels [24], it could be the case that our results are showing positive immunoreactivity to the $P2Y_6$ receptor present in the vessels that can modify the blood flow in this area.

Concerning the trabecular meshwork, our results, indicating the presence of $P2Y_1$ $P2Y_2$ and $P2Y_4$ in the rat slices, cope quite well with the results described recently by Crosson and co-workers [6]. They also described the presence of $P2Y_{11}$ in a human trabecular meshwork cells line [6]. We were not able to see a positive immunoreactivity against this receptor in the rat eye slices.

P2Y receptors in the retina

The distribution in the retina concerning the P2Y receptor is complicated, mainly due to the great cellular heterogeneity. The presence of $P2Y_2$ receptors has been demonstrated in the retinal pigmented epithelium (RPE) in situ hybridisation [12], and functional studies [11]. These studies fit well with our results, which indicate strong immunoreactivity to the $P2Y_2$ receptor. The role of this $P2Y_2$ receptor seems to be very interesting from the therapeutic point of view The presence of this receptors on the apical membrane of RPE cells is very important for the re-absorption of fluids present in the inter-retinal space [25]. This fact has invited us to think about the possibility of using selective $P2Y_2$ agonists for the treatment of pathologies such as the retinal detachment.

In the neural retina, and comparing our $P2Y_2$ results with others previously published, we were unable to see labelling in other retinal areas apart from the photoreceptors. This is in clear contrast with the results of Cowlen et al. [11], who described the presence of $P2Y_2$ mRNA in the inner nuclear layer and ganglion cell layer.

The other P2Y receptor which showed imunoreactivity in the neural retina was the $P2Y_6$ receptor, which was present at the synaptic location (the plexiform layers) as indicated by the co-localisation with synaptophysin. Only $P2X_1$ receptors have been described as associated to the plexiforms layers in the retina; these include $P2X_1$ [26–29].

Vinmentin antibody is a useful tool to identify Müller cells in the retina [30]. In this sense, we have been able to identify $P2Y_4$ and $P2Y_6$ co-localisation with vimentin in retinal slices, indicating the presence of these two purinoceptors subtypes in Müller cells. Reports about the presence of P2Y receptors in glial cells have been done [31, 32]. The existence of a P2Y receptors in Müller cells produces the typical increase in the cytosolic Ca^{2+} levels [33]. Recently, Bringmann and co-workers [34] have demonstrated how P2Y receptors can modify physiological

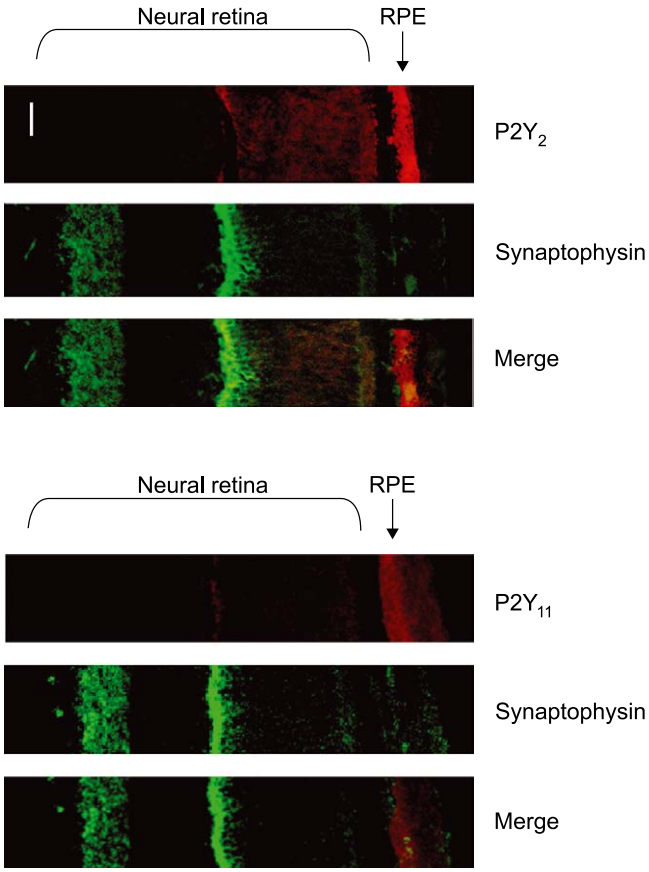

Figure 4. Presence of $P2Y_2$ and $P2Y_{11}$ in the RPE. Labelling to the $P2Y_2$ receptor was obtained in the retinal pigmented epithelium (upper pictures). Combination with synaptophysin labelling permits a better identification of the retinal areas. $P2Y_{11}$ receptors were also present in the RPE as observed in the three lower pictures. Scale bar, 10 μm.

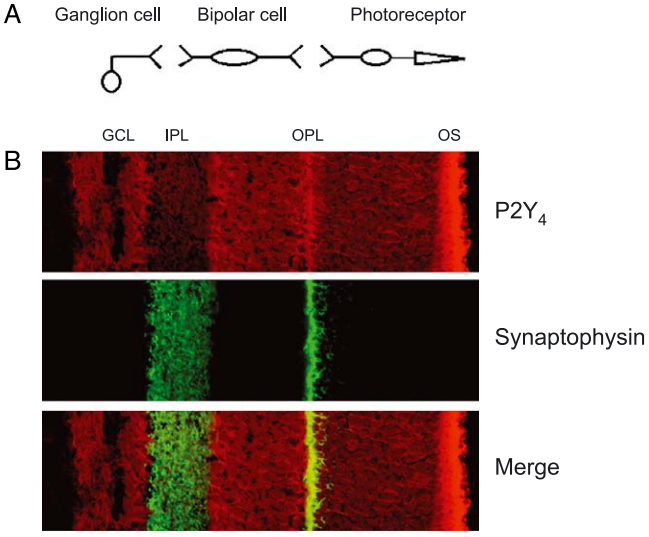

Figure 5. Distribution of $P2Y_4$ receptors in the retina. (A) Schematic diagram showing the main cells in the retina, the synapses, and how they are related. (B) The presence of $P2Y_4$ receptors in the retina. *Upper panel*, labelling with the $P2Y_4$ receptor antibody presenting positive staining in the external segments of photoreceptors (OS), outer plexiform layer (OPL) and ganglion cell layer (GCL). Mid panel, synaptophysin staining labelling the synaptic layer outer plexifom layer (OPL) and inner plexiform layer (IPL). Lower panel, combination of the upper and mid panels.

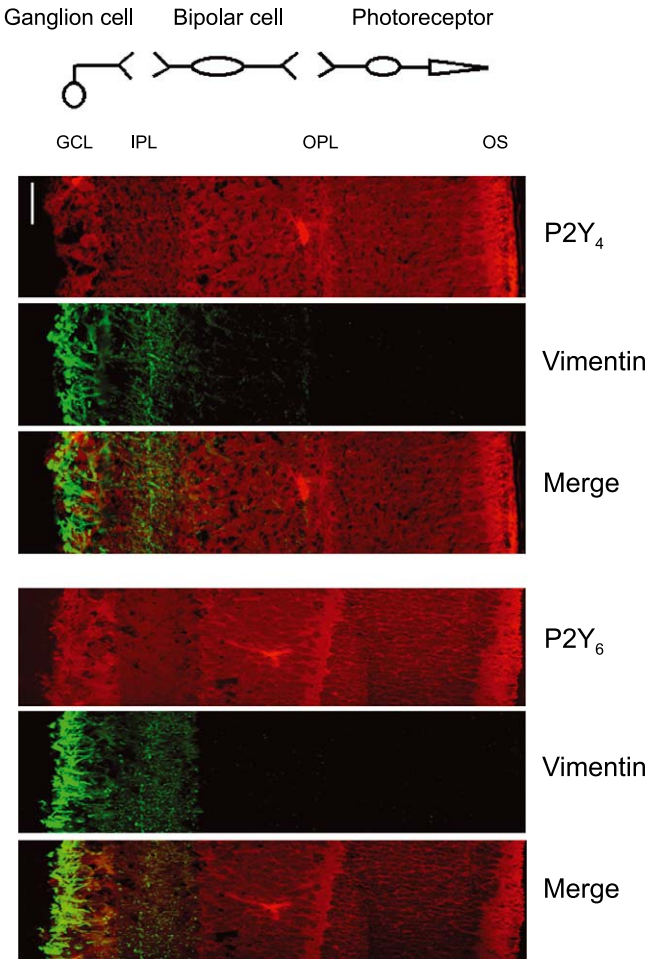

Figure 6. Study on the co-localisation of $P2Y_4$ and $P2Y_6$ receptors with vimentin (Müller cells). Upper pictures represent the labelling with the $P2Y_4$ receptor antibody (upper), with the vimentin (mid) antibody and a combination of both (lower). Lower pictures represent the labelling of $P2Y_6$ receptors (upper), vimentin (mid) and a combination of both (lower). Scale bar, 10 μm.

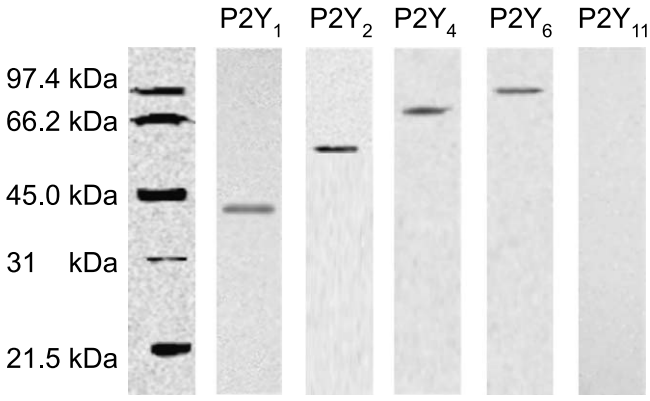

Figure 7. Western blot for the P2Y receptors in corneal extracts. Analysis of the P2Y receptors present in the cornea by Western blot analysis following the protocol described in Materials and methods. The molecular weights of the identified bands fit quite well with those described in the literature.

processes such as K^+ turnover in Müller cells, which is a crucial aspect in the neurophysiology of the retina.

Very recently, Fries and co-workers [35] described the expression of P2Y receptors in the rat retina. The use of in situ hybridization techniques demonstrated the presence of $P2Y_1$, $_4$ and $_6$ in the inner layers of the retina. We have similar results with the $P2Y_4$ and $P2Y_6$ receptor antibodies; nevertheless, we do not get labelling with the $P2Y_2$. Also, and in clear contrast with the results presented by these authors, we do not observe labelling with the $P2Y_1$ and $P2Y_2$ in the ganglion cell layer but we do find the $P2Y_4$ receptor. This group also describes immunoreactivity to the $P2Y_1$ and $P2Y_4$ in the inner plexiform layer. In our case, the only receptor located in this retinal area is the $P2Y_6$. The differences among our results and the ones described by Fries et al., can be, in part, due to the strain and age of the animals (they used Brown Norway, we used Wistar; they used adults, we used young animals). Changes in the results can also be due to the methodologies used by both groups, which differ slightly.

In summary, P2Y receptors are widely distributed in the eye. Some of the receptors were identified as P2Y although they have not been fully characterised to know which of the P2Y subtype is present in each area. We hope that this work will help researchers to apply better pharmacological tools when investigating these receptors in the different ocular structures.

Acknowledgements

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