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RESEARCH PAPER

Silencing of $P2Y_2$ receptors reduces intraocular pressure in New Zealand rabbits

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BACKGROUND AND PURPOSE

P2 receptors are involved in the regulation of ocular physiological processes like intraocular pressure (IOP). In the present study, the involvement of $P2Y_2$ receptors in the hypertensive effect of nucleotides was investigated by use of antagonists and of a siRNA designed for the $P2Y_2$ receptor.

EXPERIMENTAL APPROACH

Agonists of the $P2Y_2$ receptor a as well as P2 antagonists were applied to eyes of New Zealand rabbits, and the changes in IOP were followed for up to 6 h. Cloning of the $P2Y_2$ receptor cDNA was done using a combination of degenerate reverse transcription PCR (RT-PCR) and rapid amplification of cDNA ends (RACE). siRNA was synthesized and tested by immunohistochemistry.

KEY RESULTS

Single doses of 2-thioUTP, UTP-γ-S and UTP increased IOP. This behaviour was concentration-dependent and partially antagonized by reactive blue 2. Silencing the $P2Y_2$ receptor was observed in the ciliary body by immunohistochemistry labelling, where a reduction in the immunofluorescence was observed. This reduction in the expression of the $P2Y_2$ receptor was concomitant with a reduction in IOP, which was measurable 24 h after treatment with the siRNA, maximal after 2 days, followed by a slow increase towards control values for the following 5 days. Application of the $P2Y_2$ agonists after pretreatment of the animals with this siRNA did not produce any change in IOP.

CONCLUSIONS AND IMPLICATIONS

$P2Y_2$ receptors increase IOP in New Zealand rabbits. The application of a siRNA for this receptor significantly reduced IOP, suggesting that this technology might be used for the treatment of glaucoma.

Abbreviations

2-thioUTP, 2-thiouridine 5′ triphosphate; IOP, intraocular pressure; MRS2179, 2′ deaoxy-N6-methyladenosine 3′,5′-biphosphate; MRS2578, *N*,*N*″-1,4-butanediyl bis [*N*′-3(isothiocyanatophenyl)thiourea]; PPADS, pyridoxalphoshate-6-azophenyl-2′,4′-disulphonic acid; RB-2, reactive blue 2; siRNA, small interference RNA; UTP-γ-S, uridine 5′(γ-thio)-triphosphate

Introduction

The aqueous humour is a transparent fluid present inside the eye that provides nutritional and mechanical support to permit this structure to be active and functional (Sears and Mead, 1983). The right balance between the production and drainage of the aqueous humour is critical to keep a normal intraocular pressure (IOP) and to permit normal vision (Davson, 1993).

Many different substances have been investigated for their ability to modulate and control aqueous humour dynamics (Pintor, 2009). Amongst these molecules, nucleotides

are emerging as relevant to the ocular physiology, both on the ocular surface and in internal parts of the eye (Guzman-Aranguez *et al.*, 2007; Crooke *et al.*, 2008). Within the eye, nucleotides can exert either an increase in IOP or a decrease depending on the receptor that is stimulated (Pintor and Peral, 2001). For instance, α,β-me ATP and β,γ-me ATP can decrease IOP by acting through ionotropic P2X receptors present in the cholinergic terminals that innervate the ciliary body (Peral *et al.*, 2009). On the other hand, adenosine tetraphosphate (Ap4) and diadenosine tetraphosphate (Ap_4A) can also reduce IOP by activating metabotropic $P2Y_1$ receptors (Pintor *et al.*, 2004a; Soto *et al.*, 2005). It is clear therefore that IOP can be manipulated by using nucleotides that act on a variety of different purine receptors. This approach to produce a reduction in IOP is relevant since most of the compounds designed to treat glaucoma are focused on reducing IOP.

Apart from those P2 receptors that reduce IOP, there are others, mainly the P2Y subtype, that increase IOP as previously indicated (Peral *et al.*, 2009). This implies that if the reduction of the abnormally elevated pressure present in many glaucoma patients is going to be approached by nucleotide administration, it is important to bear in mind not only those P2 receptors that reduce IOP but also those that increase it.

It would be interesting to investigate whether or not the application of a P2 antagonist blocks those that increase IOP without affecting those that reduce it. Nevertheless, the absence of selective antagonists (Abbracchio *et al.*, 2006; Burnstock, 2006) and the abundance of P2 receptors within the eye makes this possibility really difficult (Pintor *et al.*, 2004b).

Independent of the difficulties regarding the identification of P2Y receptors present in the eye, $P2Y_2$ receptors have been found in the ciliary body (Shahidullah and Wilson, 1997; Farahbakhsh and Cilluffo, 2002; Cowlen *et al.*, 2003) as well as in the trabecular meshwork (Crosson *et al.*, 2004; Chow *et al.*, 2007). These receptors are involved in the modulation of aqueous humour dynamics (Fleischhauer *et al.*, 2001; Soto *et al.*, 2004).

RNAi technology has proven to be an efficient tool to suppress the expression of targeted genes *in vitro* and, more recently, *in vivo* (Behlke, 2006). Ocular delivery of siRNAs has also been performed to investigate gene function and new treatments in ocular diseases (Andrieu-Soler *et al.*, 2006; Campochiaro, 2006). Indeed, it has been possible to demonstrate that the selective silencing of α- and β-adrenoceptors can reduce IOP, suggesting that they may be used for the treatment of the ocular hypertension associated with glaucoma (Mediero *et al.*, 2009).

The present experimental work describes the existence of a $P2Y_2$ receptor in normotensive New Zealand white rabbits that produce an elevation in IOP. This hypertensive effect can be partially blocked by non-selective P2 antagonists and is strongly abolished by the application of a siRNA designed for the $P2Y_2$ receptor.

Methods

Animals

Male New Zealand White rabbits, one and a half years old and weighing an average of 3 kg were used for all the experiments. The animals were kept in individual cages with free access to food and water. The rabbits were kept in controlled 12 h day–night cycles to avoid circadian effects in IOP measurements. Animals for pharmacological studies were used once a week to allow them to recover for the next nucleotide application. Rabbits used for siRNA treatments were discarded after the application of the silencing agent. This study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and also all animal care and experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC).

Intraocular measurements

The effects of the different nucleotides on IOP were measured by means of a Tonopen contact tonometer supplied by MENTOR (Norwell, MA, USA). All the measurements were performed at the same time of day in order to avoid undesirable IOP variations. The Tonopen makes five consecutive IOP measurements before it provides the mean and the error of the measurement. At any given time, IOP is measured four times, and the IOP values obtained are transformed into mean ± SD. The substances were applied topically (10 μL) and in unilateral fashion, but the contralateral eye received the same volume of saline solution. Since the application of the tonometer may produce some discomfort in the animals, the corneas were lightly anaesthetized by dropping 10 μL of 1:10 (v/v) of oxibuprocaine/tetracaine (4 and 1 mg, respectively) from CUSI labs (Barcelona, Spain). Two measurements were taken at 30 min intervals between them, before any substance or saline was added. IOP was measured at 0.5, 1, 2, 3, 4, 5 and 6 h after application of the test substance.

Pharmacological studies

The nomenclature of all receptors described in this study follows that of Alexander *et al.* (2011).

$P2Y_2$ agonists tested for time course studies were assayed at single doses of 100 μM (10 μL). These compounds were also taken for a dose–response curve analysis. Concentrations ranging from 10^{-10} to 10^{-3} M were assayed and plotted versus the IOP value. The maximal effect (IOP highest value) was obtained 3 h after the drug application except for UTP, which presented two maxima, one at 2 h and the other at 5 h.

When the purinoceptor antagonists were used, pyridoxalphoshate-6-azophenyl-2′,4′-disulphonic acid (PPADS), suramin and reactive blue 2 (RB-2) were applied at 100 μM 30 min before the application of the antagonists (at a maximal dose of 10 μM in 10 μL). MRS2179 and MRS2578 were tested at 1 μM, 30 min before the application of the agonists. The contralateral eye received the same volume of vehicle, and it was taken as the control value.

When the agonists 2-thioUTP, UTP-γ-S and UTP were assayed during the application of the $P2Y_2$ siRNA, these compounds were applied at 100 μM (10 μL) following the same protocol as before but they were applied at the time when the effect of the siRNA was maximal (48 h after the instillation of the siRNA).

Cloning and siRNA design

The rabbit $P2Y_2$ receptor cDNA was cloned using a combination of degenerate reverse transcription PCR (RT-PCR) and rapid amplification of cDNA ends (RACE).

Total RNA was extracted from an established rabbit corneal epithelial cell line (SIRC, Statens Seruminstitut Rabbit Cornea) using the RNeasy Mini Kit (Qiagen, Barcelona, Spain). For first-strand cDNA synthesis, 5 μg of total RNA was retrotranscribed using the SuperScript III Reverse Transcriptase and oligo $(dT)_{20}$ (Invitrogen, Paisley, UK). The initial PCR was performed with a pair of degenerate primers based on two highly conserved regions of $P2Y_2$ receptor sequence (forward, 5′-TGCAAGCTGGTGCGYTTCCTYTTCTA-3′ and reverse, 5′-AGYCTCTGCCCWGCCAGGAAGTAGAG-3′). The PCR amplification was performed in a 50 μL volume with 2 μL of cDNA, 1X PCR buffer, 2 mM $MgCl_2$, 200 μM each dNTPs, 0.6 μM of each primer and 0.025 $U{\cdot}\mu L^{-1}$ of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA). The thermal cycling conditions for PCR were 95°C for 5 min; five cycles of 95°C for 45 s and 72°C for 2 min each; five cycles of 95°C for 45 s, 70°C for 45 s and 72°C for 2 min each; and 25 cycles of 95°C for 45 s, 64°C for 45 s and 72°C for 2 min each. The PCR products of the expected size were extracted from 1.5% low-melt agarose gels with the QIAquick Gel Extraction Kit (Qiagen), cloned with TOPO TA Cloning Kit (Invitrogen), and then sequenced. After sequencing, a new pair of primers was synthesized (forward, 5′-TCAACGA GGACTTCAAGTAYGT-3′ and reverse, 5′-CTGATACAAGTG AGGAAGAGGAT-3′) and used to obtain contiguous sequence information. PCR amplification was performed in a 50 μL volume with 2 μL of cDNA, 1× PCR buffer, 2 mM $MgCl_2$, 200 μM each dNTP, 0.4 μM of each primer and 0.025 $U{\cdot}\mu L^{-1}$ of AmpliTaq Gold® DNA polymerase (Applied Biosystems). The thermal cycling conditions for PCR were 95°C for 5 min; 10 cycles of 95°C for 45 s, 60°C for 45 s (–1°C per cycle) and 72°C for 1 min each; 30 cycles of 95°C for 45 s, 50°C for 45 s and 72°C for 1 min each; and one cycle of 72°C for 7 min each. To obtain the 5′-end of the coding sequence of rabbit $P2Y_2$ receptor cDNA, we used a FirstChoice RLM-RACE Kit (Applied Biosystems). In brief, 10 μg of total RNA was dephosphorylated, decapped and ligated to the 5′-RACE adaptor. Ligated RNA was reverse transcribed with random decamers and amplified by nested PCR. The first round of PCR was performed with a rabbit $P2Y_2$ receptor-specific reverse primer (5′-AGTGGTCGCGGGCGTAGTAGTAG-3′), and a forward adaptor primer provided in the kit. One microlitre of the PCR product obtained was used as a template for a second round of PCR. The second round of PCR was performed with a new rabbit $P2Y_2$ receptor-specific reverse primer (5′-ACACGGCCAGGTGGAACATGTA-3′) and an inner forward adaptor primer provided in the kit. The PCR products of the expected size were purified from agarose gels, cloned and sequenced. DNA sequencing was performed by the Unidad de Genómica (Parque Científico de Madrid-Universidad Complutense, Madrid, Spain). The nucleotide sequences were compared by searching the GenBank databases with the BLAST program. Alignment of amino acid sequences were performed with the Clustal W2 program (Larkin *et al.*, 2007) using default parameters.

To design $P2Y_2$ receptor-specific siRNA duplex, the rabbit $P2Y_2$ receptor coding sequence (GenBank EU886321) was submitted to the Ambion siRNA target Finder Web site (http://www.ambion.com/techlib/misc/siRNA_finder.html) for siRNA prediction. Nucleotide sequence of the siRNA target site chosen was as follows: 5′-AACCTGTACTGCAGCATC CTC-3′ (nucleotides 528–548). siRNA molecule was obtained from Applied Biosystems, in annealed and lyophilized forms, and was suspended in sterile saline (0.9% NaCl) before use.

In vivo *silencing experiments*

We determined the effects of silencing $P2Y_2$ receptors of New Zealand rabbit eyes by applying the siRNA topically. In six animals, siRNA was applied to the cornea in one single eye along four consecutive days (10 nmol 0.9% NaCl drops, 40 μL day^{-1}). The contralateral eyes were treated with sterile 0.9% NaCl. IOP experiments were performed using a design where the person doing the experiment was unaware of the treatments: no visible indication was given to the experimenter as to the applied solution (agent or vehicle). IOP was measured for 10 days following the same protocol as described for nucleotides above. Some of the experiments were repeated, and after the minimum IOP was reached, animals were killed, and both eyes were collected and used for immunohistochemical analysis.

RNA extraction and RT-PCR and qPCR

Ciliary process samples were collected at 24, 48 and 72 h after the first siRNA/sterile 0.9% NaCl (control) instillation, and total RNA was isolated using the RNeasy Mini kit (Qiagen), according to the protocol. The amount of total RNA isolated was quantified using the Quant-iT RiboGreen® RNA kit (Invitrogen). For first-strand cDNA synthesis, 1 μg of total RNA was retrotranscribed using High Capacity cDNA RT kit with random hexamer primers (Applied Biosystems). qPCR was performed in duplicate using the Quantitect SYBR Green Kit (Qiagen) with gene-specific PCR primers ($P2Y_2$ forward, 5′-TGGAGCCGTCTCTAACCCTGA-3′ and $P2Y_2$ reverse, 5′-GCTGGCACGCTGAACCAGTA-3′) on an ABI Prism 7300 Real-Time PCR System (Applied Biosystems). Non-template and non-reverse transcribed controls were included in all experiments. Analysis of the melting curves and agarose gel electrophoresis confirmed the specificity of PCR and the absence of primer–dimers. ATP5B gene (ATP5B forward, 5′-GAAGTGCAAGGCAGGAAGAC-3′ and ATP5B reverse, 5′-AATTTTGATAGGCGCACCAG-3′) was used as internal control to normalize mRNA relative expression. qPCR data analysis was performed by $2^{-\Delta\Delta Ct}$ method, once it had been confirmed that the amplification efficiency of $P2Y_2$ and ATP5B primers pairs was similar and close to a value of 2 (Livak and Schmittgen, 2001).

Western blot analysis

Protein extracts from each sample (45 mg of protein) were subjected to 10% SDS-polyacrylamide gels and were transferred to nitrocellulose membranes (Amersham-Pharmacia-Biotech, Buckinghamshire, UK). Thereafter, membranes were blocked and incubated overnight in the primary antibody appropriately diluted in PBS containing 2% skimmed milk and 0.05% Tween-20. The dilutions of primary antibodies were as follows: anti-$P2Y_1$, 1:200, anti-$P2Y_2$, 1:500 and anti-$P2Y_4$, 1:200. The primary antibodies were removed, and the blots were extensively washed with PBS/Tween-20. Blots were then incubated for 1 h at room temperature with the secondary antibody (anti-rabbit IgG coupled to horseradish peroxidase, from Sigma, St. Louis, MO) at 1:1000 dilution in 5%

(w/v) skimmed milk powder dissolved in PBS/Tween-20. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence detection system (Amersham-Pharmacia-Biotech). Films were scanned and a densitometric analysis was performed using Kodak GL 200 Imaging system and Kodak Molecular Imaging software (Kodak, Rochester, NY).

Enucleation and sectioning

Rabbits treated with the protocols previously described regarding siRNA application were killed with an overdose of sodium pentobarbital, and the eyes (treated and controls) were enucleated. After enucleation, the eyes were immediately fixed in a solution of paraformaldehyde 4% for 24 h. Then, the anterior segment was dissected in quadrants. Each quadrant containing ciliary body was cut into sections by means of a cryostat (Leica CM 1850; Leica Microsystems, Meyer Instruments Inc., Houston, TX). These cryostat sections of the ciliary processes were mounted on 3-aminopropyltriethoxysilane-treated slides for immunofluorescence.

Immunohistochemistry

Immunofluorescent staining was performed to evaluate expression and location of the $P2Y_2$ in the ciliary processes of New Zealand rabbits. Also, we showed that siRNA was effective against $P2Y_2$ in the treated eyes. After several washes in PBS and pre-incubation in PBS with 3% blocking serum for 1 h, the sections were incubated for 2 h at room temperature with the primary antibody, a mouse polyclonal antibody raised against a full-length recombinant $P2Y_2$ (1:100). In a second step, after several washes with PBS, the sections were incubated in a dark chamber with the secondary antibody goat anti-mouse IgG-FITC (1:500) for 1 h at 37°C. After several washes, the samples were coverslippped with Vectashield (Vector Labs, Peterborough, UK) and observed under a confocal microscope (Axiovert 200 M; Carl Zeiss Meditec GmbH, Jena, Germany), equipped with a PASCAL confocal module (LSM 5; Zeiss, Jena, Germany).

All images were analysed by the accompanying PASCAL software (Carl Zeiss).

Compounds

UTP, UTP-γ-S and 2-thioUTP, MRS2179 and MRS2578 were purchased from Tocris (Bristol, UK). PPADS, suramin and RB-2 were obtained from Sigma. Other reagents were of analytical grade from Merck (Darmstadt, Germany).

Statistical analysis

All data are presented as the mean ± SD. Significant differences were determined by ANOVA. The plotting and fitting of the dose–response curves were carried out by the computer programme GraphPad Prism 4.0c (GraphPad Software Inc., La Jolla, CA, USA).

Results

Single concentration application

The effects of different $P2Y_2$ synthetic nucleotide agonists on IOP were tested in groups of eight normotensive rabbits.

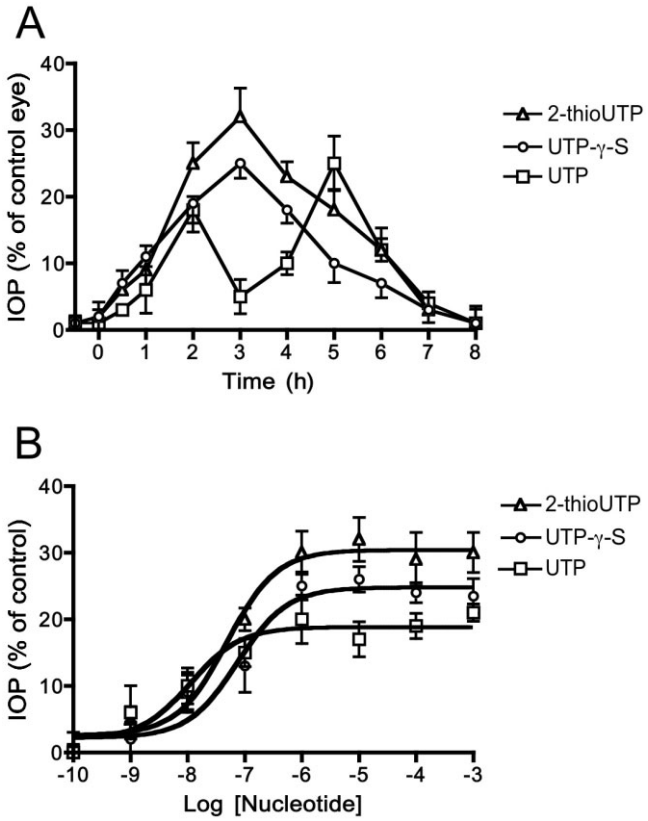

Figure 1

Effect of $P2Y_2$ agonists on IOP. (A) Effect of 2-thioUTP, UTP-γ-S and UTP (100 μM, 10 μL) on IOP in New Zealand White rabbits. The values are the mean ± SD of eight independent experiments. (B) Concentration–response curve for 2-thioUTP, UTP-γ-S and UTP ranging from 10^{-10} to 10^{-3} M. The values are the mean ± SD of eight independent experiments.

Since basal IOP can vary between animals, all the data were normalized to their initial, corresponding IOP values (IOP mean 15.4 ± 4.3 mmHg). Single doses of 100 μM (10 μL) 2-thioUTP, UTP-γ-S and UTP were assayed, and the changes in IOP were studied for 8 h after the application. 2-thioUTP and UTP-γ-S produced a gradual increase in IOP, which was maximal between 2 and 3 h after the instillation of the tested substance (Figure 1A). 2-thioUTP elevated IOP the most with an increase of 32 ± 11% over control ($P < 0.005$), followed by UTP-γ-S with an increase of 25 ± 5% over control ($P < 0.02$). UTP produced two peaks of IOP, the first one, 2 h (18 ± 5% of increase over control), and the second 5 h (25 ± 11% of increase over control), after its application (Figure 1A).

Concentration–response analysis

The three nucleotides induced concentration-dependent effects when assayed at concentrations ranging from 10^{-10} to 10^{-3} M. Among them, 2-thioUTP was the one producing the most robust increase in IOP, followed by UTP-γ-S and UTP, respectively (Figure 1B). The pD_2 values were: 2-thioUTP, 7.31 ± 0.39 (EC_{50} = 48.8 nM), UTP-γ-S 7.12 ± 0.62 (EC_{50} = 0.74 nM) and UTP 7.94 ± 0.74 (EC_{50} = 11.2 nM) (n = 8). For the UTP

experiments, peak increases in IOP were measured 2 h after its application ($n = 8$; see Discussion).

Effect of P2 receptor antagonists

In order to identify the receptor mediating the rise in the IOP, the ability of different non-selective antagonists and two selective antagonists for the $P2Y_1$ and $P2Y_6$ to abolish the effect of these nucleotides was investigated.

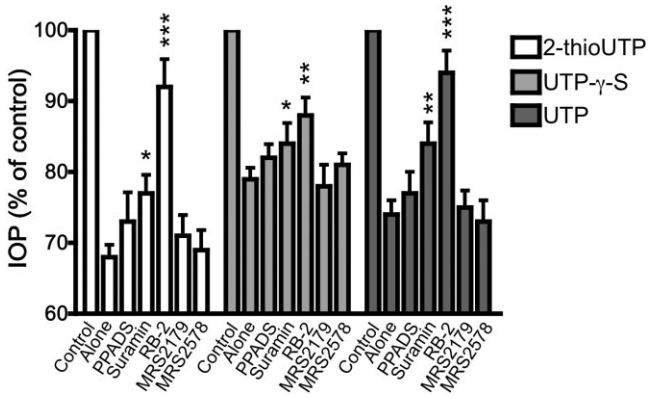

Figure 2

Effect of antagonists on $P2Y_2$ receptor activation. The antagonistic ability of the non-selective P2 antagonists suramin, PPADS and reactive blue 2 (all at 100 μM, 10 μL, see Methods) and the $P2Y_1$ antagonist MRS 2179 and $P2Y_6$ antagonist MRS 2578 (1 μM, 10 μL) were assayed on 2-thioUTP, UTP-γ-S and UTP. The values are the mean ± SD of eight independent experiments. ***$P < 0.001$ versus agonist alone; **$P < 0.005$ versus agonist alone; *$P < 0.05$ versus agonist alone.

As it can be seen in Figure 2, neither MRS2179 ($P2Y_1$ antagonist) nor MRS2578 ($P2Y_6$ antagonist) were able to prevent the hypertensive effect that the three nucleotides presented in the absence of the antagonists. On the other hand, of the non-selective P2 antagonists, PPADS was unable to reverse the effect of the three nucleotides, whereas suramin slightly, but significantly, reduce the effect of the nucleotides. This antagonist reduced IOP in the case of 2-thioUTP from 32 ± 3% to 23 ± 5% ($P < 0.05$, $n = 8$), UTP-γ-S from 21 ± 3% to 16 ± 8% ($P < 0.05$, $n = 8$) and UTP from 26 ± 5% to 16 ± 8% ($P < 0.05$, $n = 8$). Reactive blue 2 (RB-2) was the most effective antagonist counteracting the hypertensive effect of 2-thioUTP to values of 9 ± 11% ($P < 0.001$, $n = 8$), UTP-γ-S to 12 ± 5% ($P < 0.005$, $n = 8$) and UTP to 8 ± 8% ($P < 0.001$, $n = 8$) (Figure 2).

Again, for the UTP experiments we measured the variations in IOP obtained 2 h after its application (see Discussion).

Cloning of rabbit $P2Y_2$ receptor

To determine the effect of the $P2Y_2$ receptor on IOP by siRNA technology, we cloned the rabbit $P2Y_2$ receptor cDNA. Assembly of the sequences obtained by RT-PCR and 5′-RACE yielded a sequence of 1094 bp (GenBank accession number EU886321). Protein database searches revealed that the deduced sequence corresponded highly with the amino acid sequence of the human $P2Y_2$ receptor (94% identical, Figure 3).

Safety of in vivo *siRNA delivery*

To confirm the absence of ocular changes after frequent instillations of siRNA, we performed a biomicroscopic examination of the treated eyes. Neither corneal inflammation, cataract or any other ocular alteration were observed

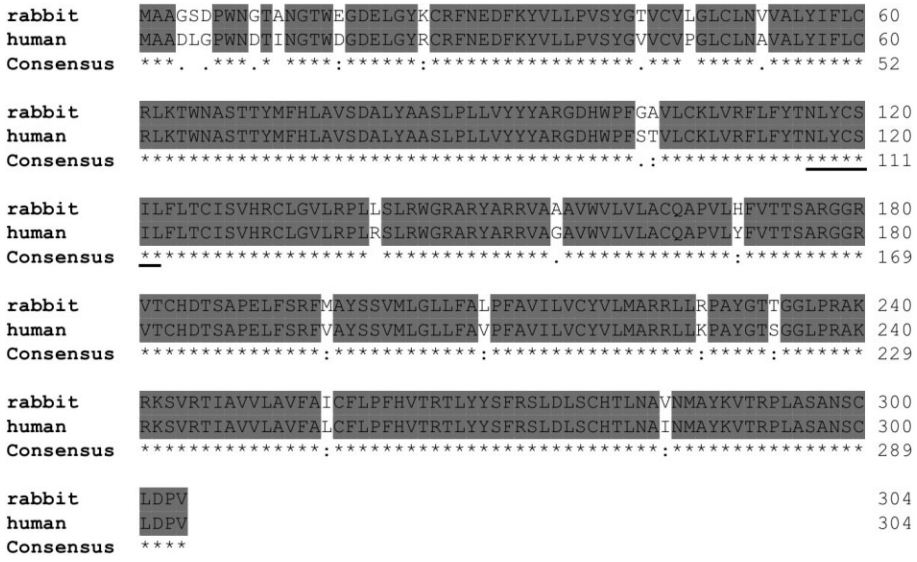

Figure 3

Sequence of the rabbit $P2Y_2$ receptor. Deduced amino acid sequence of the cloned rabbit $P2Y_2$ receptor and its alignment with the human sequence. * Represents identical residues; : represents conserved substitutions and . semi-conserved substitutions. siRNA-target sequence is indicated with solid line. These sequences are available under GenBank accession number EU886321 (rabbit) and NP_788086.1 (human).

throughout the administration of the siRNA (results not shown).

Silencing the P2Y$_2$ receptor in the rabbit ciliary body

To test the efficacy of synthesized siRNA targeting the P2Y$_2$ receptor, RT-qPCR and immunohistochemistry assays were performed.

To test the selectivity of the silencing of P2Y$_2$ receptor, we applied the P2Y$_2$ siRNA and then analysed the expression of P2Y$_1$, P2Y$_2$ and P2Y$_4$ receptors, which are present in the ciliary body (Pintor *et al.*, 2004b). As can be seen in Figure 4A, none of the P2Y receptors, apart from P2Y$_2$, was modified 48 h after the application of the siRNA. Relative quantification of the P2Y$_2$ reduction showed levels of 33 ± 6% ($P < 0.001$), compared with the unchanged values obtained for the other receptors (Figure 4B).

The siRNA-treated animals presented a marked reduction in P2Y$_2$ receptor mRNA levels 24 and 48 h after the first siRNA dose (9.12 ± 1.63% and 16.19 ± 1.86% of P2Y$_2$ mRNA remaining, respectively; $P < 0.001$) compared with the control (eye treated with sterile 0.9% NaCl) (Figure 5A). Also, P2Y$_2$ transcript levels were still diminished after 96 h of siRNA treatment (63.51 ± 1.63% of P2Y$_2$ mRNA remaining, $P < 0.01$) (Figure 5A). This reduction in mRNA at 24, 48 and 72 h was also observed when measuring the expressed P2Y$_2$ protein by Western blot. Figure 5B presents the changes in the expression of P2Y$_2$ receptors at 24, 48 and 72 h after the application of the siRNA for the P2Y$_2$ receptor compared with control (time 0). These results showed values of 45 ± 11% ($P < 0.01$) at 24 h, 20 ± 15% ($P < 0.001$) at 48 h and 48 ± 21% ($P < 0.001$) at 72 h (Figure 5B).

The analysis of P2Y$_2$ receptor in the ciliary body of New Zealand white rabbits showed that there was an intense labelling in both pigmented and non-pigmented epithelia cells as it can be observed in the upper panel of Figure 6.

Forty-eight hours after the application of the siRNA against the P2Y$_2$ receptor, it was possible to observe a clear reduction in the fluorescence intensity, suggesting a reduction in the presence of P2Y$_2$ receptors (Figure 6, lower panel). The relative quantification of the reduction in terms of fluorescence intensity demonstrated a reduction of 85% of the signal compared with the untreated animals ($P < 0.005$; $n = 6$).

To see whether this reduction was concomitant with changes in the IOP, the IOPs of animals treated with the siRNA against the P2Y$_2$ receptor were monitored for 7 days. As presented in Figure 5A, there was a robust reduction in IOP, which was present 24 h after the instillation of the siRNA and was statistically significant for 5 days. The maximal reduction obtained after silencing the P2Y$_2$ receptor was 48 ± 5% compared with control (n = 6). This and similar values were maintained for 1–2 days, with the IOP remaining low for three more days (with a mean of 33% of reduction) and then returning slowly towards control values (Figure 5A).

In order to further confirm the selectivity of the siRNA, a scramble siRNA was also assayed. As observed in Figure 5A, after treatment with the scramble siRNA, rabbit IOP did not significantly change during the time of the experiment.

Single doses of either 2-thioUTP, UTP-γ-S or UTP (all at 100 μM, 10 μL), 48 h after the instillation of the siRNA for the P2Y$_2$, were unable to produce hypertensive effects these compounds depict in untreated animals, thus indicating their preferential action by a P2Y$_2$ receptor.

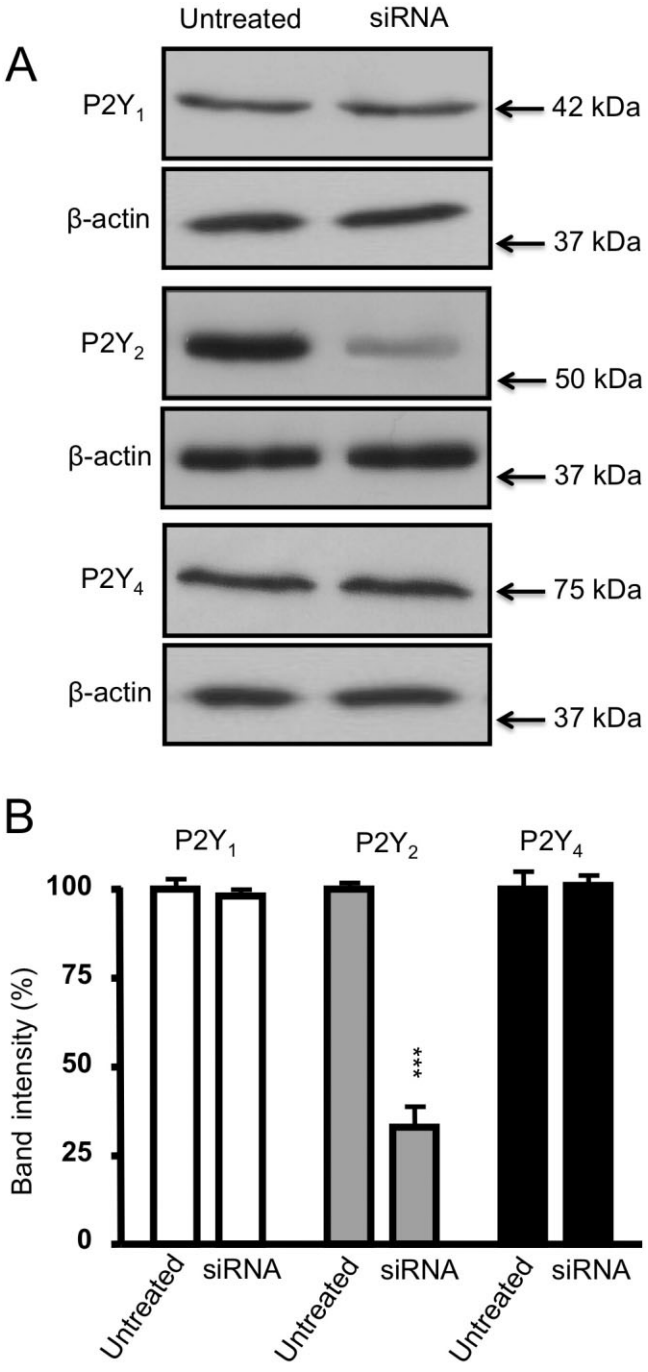

Figure 4

Selective effect of siRNA on P2Y$_2$ receptors. (A) Western blot analysis showing the effect of the siRNA for the P2Y$_2$ on P2Y$_1$, P2Y$_2$ and P2Y$_4$ receptors present in the rabbit cilliary body (see Methods for details). (B) Relative quantification of the Western blot band intensities before and after the application of the siRNA for the P2Y$_2$ receptor. Values are the mean ± SD of three independent experiments. ***$P < 0.005$ versus control.

To verify the selectivity of the siRNA in the treated eye, the silencing was followed in the treated eye and simulta-

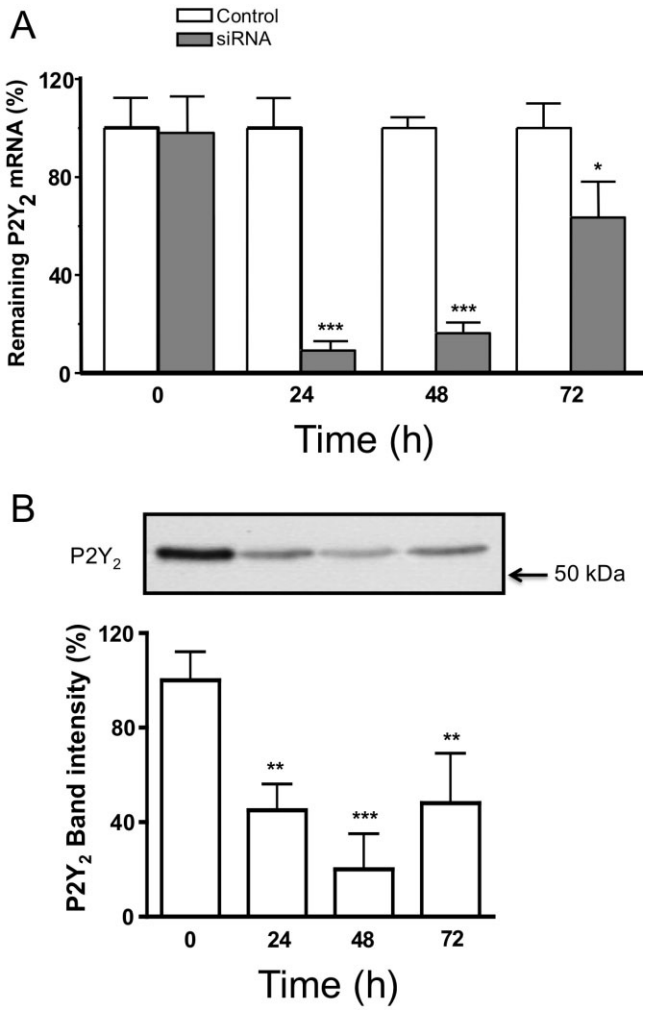

Figure 5

Quantification of $P2Y_2$ receptor by Western blot and mRNA levels by RT–qPCR analysis. (A) $P2Y_2$ receptor mRNA levels, in animals treated with siRNA, normalized to ATP5B gene and relative to control levels (set to 100%). Each data point represents mean percentage ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, compared with control levels. (B) $P2Y_2$ receptor expression measured by Western blot at the indicated times. Each data point represents mean percentage ± SD of three independent experiments. **$P < 0.01$ and ***$P < 0.001$, compared with control levels (day 0).

neously in the contralateral eye, which was instilled with vehicle. As observed in Figure 7C, 2-thioUTP had an almost non-existent effect in the treated eye, while in the contralateral eye, it effectively increased IOP.

Discussion

The present experimental work describes the effect of three $P2Y_2$ agonists, 2-thioUTP, UTP-γ-S and UTP, on IOP in New Zealand white rabbit. The three pyrimidine nucleotides increased IOP in a concentration-dependent manner; an effect that could be partially antagonized by non-selective P2 antagonists. The confirmation of the involvement of a $P2Y_2$ receptor mediating the hypertensive effects in this animal model was performed by the application of a siRNA against the rabbit $P2Y_2$ mRNA. The silencing of the receptor produced a profound and long lasting reduction in IOP.

Although the compound that mediated the strongest increase in IOP was 2-thioUTP, the compound that depicted a peculiar behaviour was the naturally occurring nucleotide UTP. This nucleotide presented an initial increase in IOP with a maximum 2 h after its administration, then a reduction in IOP and finally at 5 h another raise in IOP. The first increase corresponds to the effect of UTP; the following decrease in IOP is due to its degradation to UDP as previously demonstrated (Markovskaya *et al.*, 2008). The second rise in IOP is a question that has not been solved yet. The effect of naturally occurring mononucleotides like UTP is always limited mainly because of the action of ecto-nucleotidases. This phenomenon also occurs with ATP when assayed for its ability to reduce IOP, indicating the existence of enzymes capable of transforming these nucleotide triphosphates into their hydrolyzed products (Peral *et al.*, 2009). Therefore, we performed all our experiments, taking into account the first increase in IOP produced by UTP.

Antagonism by means of several commercially available compounds did not shed light or confirm that the receptor activated by these pyrimidine agonists was a $P2Y_2$ receptor. In our hands, neither MRS2179 ($P2Y_1$ antagonist) nor MRS2575 ($P2Y_6$ antagonist) modified the response to any of the three nucleotides. Only the non-selective antagonists and in particular RB-2 partially reversed the action of 2-thioUTP, UTP-γ-S and UTP. Nevertheless, it is interesting to note that in a previous study the hypotensive effect of UDP was reversed by PPADS, while in the present study, this antagonist did not significantly modify the effects of any of the nucleotides tested (Markovskaya *et al.*, 2008).

The eye is a relatively isolated compartment, which makes it an ideal target organ for gene therapy (Campochiaro, 2006). siRNA has been successfully achieved in animal models of ocular neovascularization and scarring using saline and lipid formulations (Reich *et al.*, 2003; Nakamura *et al.*, 2004). In the present work, we determined the effect of the $P2Y_2$ receptor on IOP through RNAi technology. siRNAs against P2 receptors have been previously used to help to understand their physiological roles (Arthur *et al.*, 2005; 2007; D'Alimonte *et al.*, 2007; Deli *et al.*, 2007; Ecke *et al.*, 2008; Glatt *et al.*, 2008; Murakami *et al.*, 2008). In our case, we demonstrated the involvement of this receptor in increasing IOP in New Zealand rabbits, but also, we revealed the possibility of using this siRNA as an agent to reduce IOP.

The use of the siRNA against the $P2Y_2$ receptor is of interest when finding new therapeutic approaches to treat the abnormal elevation in IOP present in many glaucoma patients. In this context, it has been demonstrated that patients with acute glaucoma present abnormally elevated concentrations of ATP in their aqueous humour (Zhang *et al.*, 2007). More recently, patients with primary open angle glaucoma, the most common type of glaucoma, also present increased levels of nucleotides in their aqueous humours (Castany *et al.*, 2011). It is possible that these high concentrations of nucleotides, by activating the $P2Y_2$ receptors

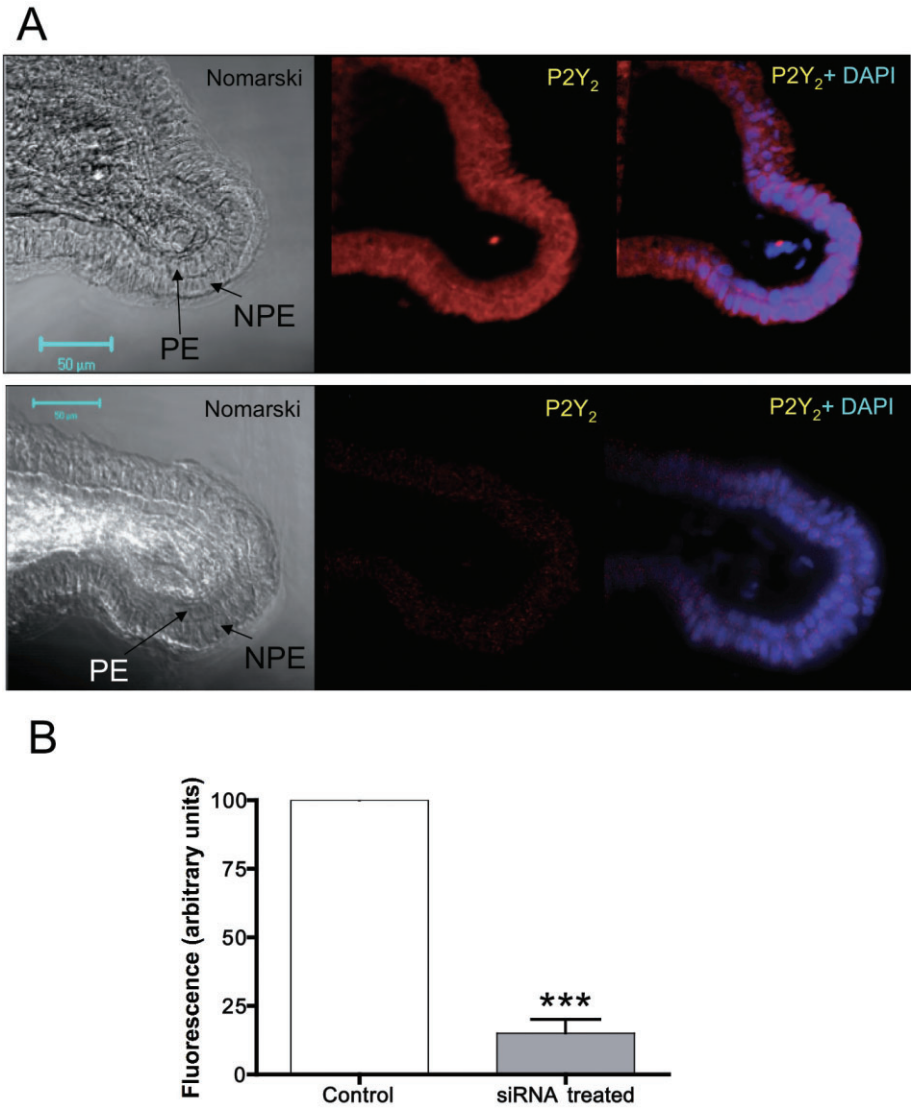

Figure 6

Immunohystochemical analysis of the $P2Y_2$ silenced by siRNA. (A) Upper panel, visualization of a ciliary process by Nomarski interferential technique, $P2Y_2$ antibody (mid picture in red) and the combination of the previous with DAPI to label nuclei. Lower panel shows the effect of siRNA on $P2Y_2$ label of a ciliary body. These are representative pictures of six independent experiments. (B) Quantification of the $P2Y_2$ fluorescent decay after the application of the siRNA expressed in arbitrary units. Values are the mean ± SD of six independent experiments. ***$P < 0.005$ versus control.

described in the present work, are responsible for the abnormal values of IOP in glaucoma patients. Hence, $P2Y_2$ receptors may be one of the factors involved in the hypertensive status of primary open angle glaucoma patients However, more experiments are needed to demonstrate the relationship between $P2Y_2$ activation and the high levels of nucleotides in glaucoma patients.

In summary, New Zealand white rabbits present $P2Y_2$ receptors that can be activated by 2-thioUTP, UTP-γ-S and UTP to produce a rise in the IOP. These hypertensive effects were abolished when the $P2Y_2$ receptor was silenced by means of a siRNA. The finding that silencing the $P2Y_2$ receptor produced a robust decrease in IOP raises the possibility that this siRNA could be used as a therapeutic agent for glaucoma.

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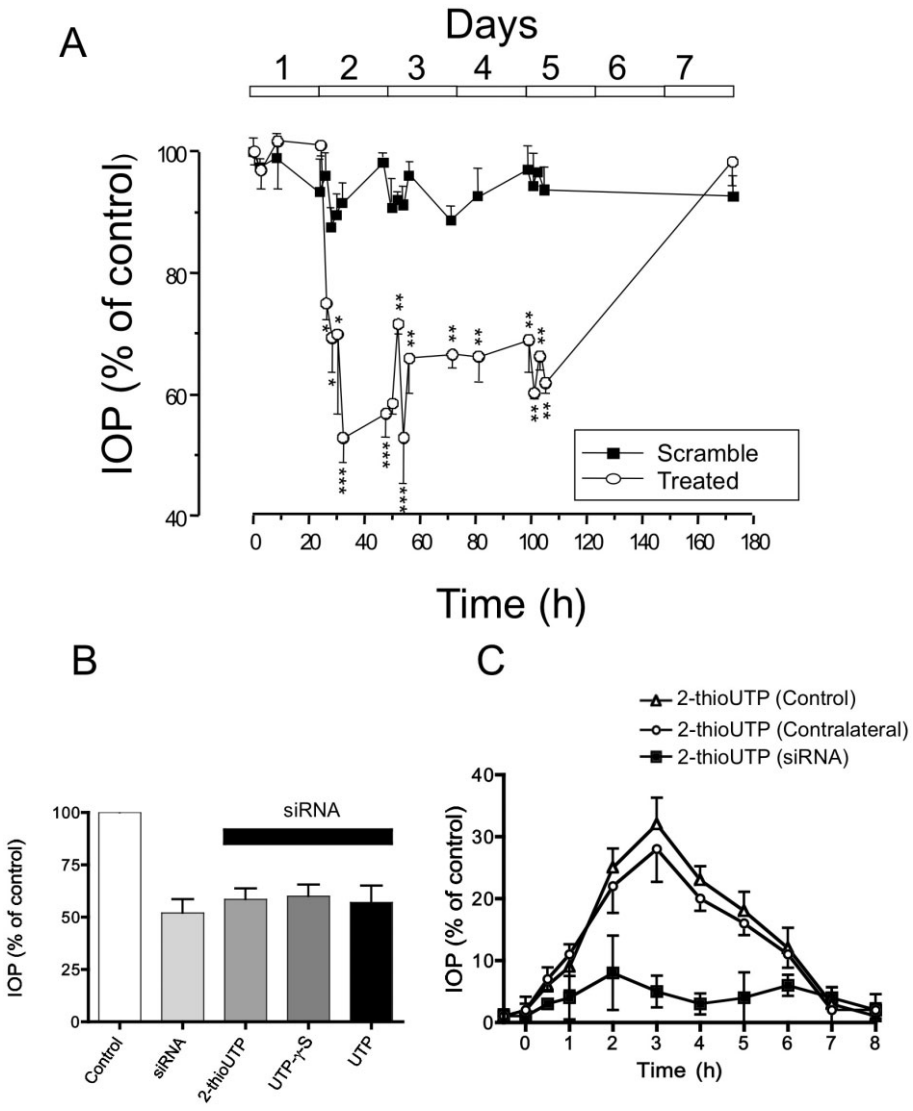

Figure 7

Effect of P2Y$_2$ silencing on rabbit IOP. (A) IOP in siRNA treated animals presented a sharp reduction of IOP, which stared 24 h after the beginning of the experiment and which remained low for about 4 days before it returned to normal values. Contralateral eyes treated with vehicle presented minor fluctuations in IOP. Values are the mean ± SD of six independent experiments. (B) Prevention of the hypertensive effect of 2-thioUTP, UTP-γ-S and UTP (all at 100 μM, 10 μL) when the nucleotides were tested after pretreatment with P2Y$_2$ siRNA (48 h after the initiation of the treatment).

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Conflicts of interest

None.

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