Diadenosine tetraphosphate contributes to carbachol-induced tear secretion

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Abstract The purpose of this study is to investigate if the cholinergic stimulation by carbachol on tear secretion is a direct process or if it is also mediated by purinergic mechanisms. Experiments were performed in New Zealand male rabbits. The amount of tear secretion was measured with Schirmer's test and then analyzed by a HPLC protocol in order to study the nucleotide levels. Animal eyes were instilled with carbachol (a cholinergic agonist), pirenzepine, gallamine and 4-DAMP (muscarinic antagonists), PPADS, suramin and reactive blue 2 (purinergic antagonists), and a P2Y₂ receptor small interfering RNA (siRNA). Tear secretion increased with the instillation of carbachol, approximately 84 % over control values 20 min after the instillation and so did Ap₄A and ATP release. When we applied carbachol in the presence of muscarinic antagonists, tear volume only increased to 4 % with atropine, 12 % in the case of pirenzepine, 3 % with gallamine, and 8 % with 4-DAMP. In the presence of carbachol and purinergic antagonists, tear secretion was increased to 12 % (all values compared to basal tear secretion). By analyzing tear secretion induced with carbachol in presence of a P2Y₂ receptor siRNA, we found that tear secretion was diminished to 60 %. The inhibition of tear secretion in the presence of carbachol and purinergic antagonists or P2Y₂ siRNA occurred with no apparent change in the tear amount of Ap₄A. These experiments demonstrated the participation of Ap₄A in lacrimal secretion process.

Keywords Carbachol · Purinergic · Nucleotides · Tear secretion · P2Y

Introduction

The tear film constitutes a moist natural barrier that separates the eye from the external media. This consistent film is formed mainly by a triplet of aqueous, mucous, and lipid layers that provide the necessary equilibrium for maintaining the health of the ocular surface [1]. The main functions of this film are to keep the ocular surface wet and well lubricated, to transfer the nutritional elements to the cornea, to eliminate foreign matter and cellular debris generated on the ocular surface by the tear flow and the blink process, and, finally, to act as the first line of defense against ocular surface infections [2].

Tear secretion is regulated by autonomic nerves that also innervate the cornea and conjunctiva [3]. In this sense, the efferent parasympathetic and sympathetic nerves innervate the lacrimal gland, the lacrimal gland secretory cells, and the lacrimal gland excretory ducts. Activation of these nerves stimulates the lacrimal gland, promoting secretion of lacrimal gland electrolytes, water, and proteins.

The main neurotransmitters that regulate tear secretion are the parasympathetic neurotransmitters acetylcholine and VIP, as well as the sympathetic neurotransmitter norepinephrine. These agonists are all stimulatory and can activate different signaling pathways conducting to stimulate tearing [4]. Although both, the parasympathetic and the sympathetic nerves innervate the lacrimal gland, the parasympathetic system predominates, both anatomically and functionally [5–8].

Indeed, the effect of muscarinic antagonists confirms a major role for parasympathetic nerves in the stimulation of lacrimal gland secretion as systemic administration of scopolamine produces dry eye in mice that is exacerbated by a desiccating environment [9]. On the contrary, intraarterial or

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intraperitoneal systemic administration of acetylcholine, pilocarpine, or other cholinergic agonists stimulates lacrimal gland protein and fluid secretion measured from the cannulated lacrimal gland excretory duct of anesthetized rabbits [10–16]. Altogether, this indicates that acetylcholine is the main transmitter controlling tear secretion.

Despite the importance of the cholinergic component in the regulation of tear secretion, it is important to emphasize that part of the interesting properties of the tears relies on the components they contain. A remarkable compound, depicting interesting physiological properties on the ocular surface is diadenosine tetraphosphate also termed as Ap_4A . This dinucleotide is present in human tears [17] and can stimulate tear secretion after a single-dose topical instillation as measured by Schirmer's test [18]. Ap_4A can activate P2X and P2Y receptors as well as its own dinucleotide receptor [19, 20]. In the eye; this dinucleotide activates mainly P2Y₂ receptors [21], modifying several physiological processes such as tear secretion, wound healing, and intraocular pressure [18, 22, 23].

The importance of Ap_4A is based not only in its ability to induce tear secretion, but its concentrations can vary in patients with dry eye, being therefore a biological marker of dry eye [24].

Diadenosine tetraphosphate is naturally released from the ocular surface as a consequence of blinking. Moreover, it has been possible to observe that this nucleotide can be released from corneal epithelial cells after mechanical shear stress stimuli [25].

Altogether, tear secretion is dually stimulated by a neural component, acetylcholine, and a non-neural component, Ap_4A , but little is known about how both substances interact in the tear secretion process. Therefore, in this experimental work, we study the relationship between the cholinergic and the purinergic components that permits the normal functioning of the ocular surface.

Materials and methods

Animals

A total of 24 male New Zealand white rabbits (2.5 ± 0.5 kg) were used along all the experimentation. The animals were kept in individual cages with free access to food and water. All the protocols described here adhere to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research, and also the experiments were carried out in accordance with the principles of UK legislation and the European Communities Council Directive (86/609/EEC).

Compounds and solutions

Carbachol (carbamylcholine), atropine, pirenzepine, galamine, and Ap_4A were purchased from Sigma, St. Louis,

USA. 4DAMP, PPADS, suramin, and reactive blue 2 were purchased from Tocris, Minneapolis, MN, USA. All compounds were prepared at 100 μ M concentration and applied at a fixed volume of 10 μ L. P2Y₂ receptor small interfering RNA (siRNA) (10 μ L/250 μ M) was purchased from Life Technology, CA, USA.

HPLC procedures

The chromatographic system consisted of a Waters 1515 Isocratic HPLC pump, a 2487 dual absorbance detector, and a Reodyne injector, all managed by the software Breeze from Waters (Milford, MA). The column was a Novapak C18 (15 cm in length, 0.4 cm in diameter) from Waters. The system was equilibrated overnight with 10 mM KH_2PO_4 , 2 mM tetrabutylammonium (TBA), 12 % acetonitrile, and pH 7.5, and detection of nucleotides was performed under isocratic conditions with the mobile phase described above at a flow rate of 2 mL/min.

After injection of 100 μ L of sample, detection was monitored at 254 nm wavelength. Peaks were identified as putative diadenosine polyphosphates, based on comparing their retention times with the ones of commercial standards. The quantification was performed by comparing the areas under the curves with those of known amounts of commercial standards.

Tear volume measurements

Tear secretion was measured by using the Schirmer's I test. The tear collection was always performed according to Van Bijsterveld criteria [26]. The Schirmer strip was placed on the temporal tarsal conjunctiva of the lower lid for 5 min with the eyes closed. The volume of tears, in millimeters of moistened strip, was recorded, and the Schirmer strips were placed in tubes (Eppendorf, Fremont, CA) containing 500 μ L of purified water (Ultrapure; Millipore, Billerica, MA). The samples were then frozen until they were analyzed by high-pressure liquid chromatography (HPLC).

Control experiments were performed by applying 10 μ L of saline solution (NaCl 0.9 %) and 5 min after that, the Schirmer strip was applied in the rabbit's lower lid for 5 min. When the experiments were performed, the same volume of the desired compound at the concentration indicated in each case was applied and 5 min after the instillation, the Schirmer strip was applied for 5 min as previously indicated. Tear secretion in each case was measured as the length of the wet strip (in mm).

Immunohistochemical studies

Immunofluorescent labeling was performed to evaluate the expression of the P2Y₂ in the lacrimal gland of New Zealand rabbits. 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 P2Y₂ (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-TRICT (1:500) for 1 h at 37 °C. After several washes, the samples were cover-slipped 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 analyzed by the accompanying PASCAL software (Carl Zeiss).

siRNA studies

To design P2Y₂ receptor-specific siRNA duplex, the rabbit P2Y₂ 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). The siRNA molecule was obtained from Applied Biosystems, in annealed and lyophilized forms, and was suspended in sterile saline (0.9 % NaCl) before use.

We determined the effects of silencing P2Y₂ receptors of New Zealand rabbit eyes by applying the siRNA topically. In eight animals, siRNA was applied to the ocular surface in the sac of the lower lid, in one single eye in four consecutive days (10 µL/250 µM). The contralateral eyes were treated with the same volume of sterile 0.9 % NaCl containing a scramble.

Statistical analysis

All data are presented as the mean±S.E.M. Statistical differences between treatments were calculated using ANOVA test and *t* test. Plotting and fitting were carried out by GraphPad Prism 5 computer program (GraphPad software).

Results

Effect of carbachol in tear secretion in New Zealand white rabbits

Carbachol, the long-lasting analog of the naturally occurring transmitter acetylcholine, was assayed in order to see its effects on tear secretion. As it is shown in Fig. 1a, a single dose of 10 µL, 100 µM carbachol induced a peak in tear secretion of 81.53±11.11 % over basal tear secretion (10 µL NaCl 0.9 %) 20 min after the application of the substance (*n*=8). This effect

Fig. 1 Effect of carbachol in tear secretion. **a** Time course of carbachol (10 µL, 100 µM) followed by 65 min. It induced an increase of 81.53±11.11 % over tear secretion compared to control rabbits, which were instilled with 10 µL saline solution. Values represent mean±S.E.M of six independent experiments. ****p*<0.001, ***p*<0.01 (two-way ANOVA with Bonferroni's posttest). **b** Concentration–response course for carbachol. It depicted a sigmoidal behavior presenting a pD₂ 5.0±0.3, which was equivalent to an EC₅₀ of 0.13 µM. Values represent the mean±S.E.M of six independent experiments. ****p*<0.001, **p*<0.05 (two-way ANOVA with Bonferroni's posttest)

was measurable during approximately 1 h, the moment when tear secretion returned to basal values (Fig. 1a).

When carbachol was tested in a broad range of concentrations (from 10⁻⁸ M to 10⁻³ M) in order to see the concentration–response behavior, it was possible to obtain a sigmoid dose–response curve. As it can be seen in Fig. 1b, carbachol sigmoidal curve obtained presented a pD₂ values of 5.0±0.3 which was equivalent to an EC₅₀ of 0.13 µM (*n*=8).

Antagonist studies

We measured effect of carbachol in the presence of muscarinic and purinergic antagonists (Fig. 2a). Carbachol produced an increase in tear secretion of 83.33±10.11 % above basal tear secretion value. Under this stimulated conditions, the effect of the acetylcholine analog was challenged by means of several muscarinic receptor antagonists. In this sense, tear secretion was inhibited 87.08±20.20 % with atropine (non-selective muscarinic receptor antagonist), 72.04±16.90 % in the case

Fig. 2 Antagonism by atropine, pirenzepine, gallamine, 4-DAMP, and purinergic antagonists (100 μ M, 10 μ L) of the response produced by carbachol. **a** Values are the mean $\pm$ S.E.M. of six independent experiments. *** p <0.001, * p <0.05 (one-way ANOVA with Dunnett's test). **b** Effect of purinergic antagonists in tear secretion. Time course of purinergic antagonists in presence of carbachol (10 μ L, 100 μ M) followed by 65 min. Values represent the mean $\pm$ S.E.M. of six independent experiments. *** p <0.001, * p <0.05 (two-way ANOVA with Bonferroni's posttest)

of pirenzepine (M1 antagonist), 86.33 ± 20.56 % with gallamine (M2 antagonist), and 91.21 ± 11.796 % with 4-DAMP (M3 antagonist) ($n=8$).

Interestingly, when a cocktail of P2 receptor antagonists (PPADS, suramin and reactive blue 2, all at 100 μ M) were tested on their ability to modify tear secretion, it was possible to demonstrate that they were able to significantly reduce carbachol-induced tear secretion reaching 80.0 % of carbachol effect (100 %, $n=8$). Interestingly, these 20 % of inhibition returned to basal secretion with a significant delay as it can be seen in Fig. 2b.

P2Y₂ receptor siRNA studies

In order to confirm the involvement of P2 receptors in the carbachol-induced tear secretion experiments with a siRNA for the P2Y₂ receptor were performed since this has been

described as an important receptor triggering the production of tear secretion as indicated in the introduction.

Rabbits were instilled in two consecutive days with siRNA and with carbachol the next day (third day from the beginning of the experiment). Tear secretion measurements were performed in three batches of animals: (a) untreated animals (scramble), (b) animals treated with carbachol, and (c) animals treated with siRNA and carbachol.

Immunohistochemical analysis demonstrated that the widespread presence of P2Y₂ receptors in the lacrimal gland clearly disappeared 48 h after the application of the siRNA when compared to the scramble (control), as it can be seen in Fig. 3.

The lack of P2Y₂ receptors also affected tear secretion induced by carbachol. As it can be seen in Fig. 4a, tear secretion was partially blocked when the presence of P2Y₂ receptors was reduced by the treatment with the P2Y₂ siRNA. The rise in tear secretion induced by carbachol in this situation was 54.8 ± 8.2 % less than when carbachol was applied alone (181.8 %) ($n=8$). Interestingly, even in the absence of any given compound, it was possible to measure a fall in tear secretion starting 24 h after the application of the P2Y₂ siRNA and lasting for another 24 more hours (Fig. 4b).

Levels of Ap₄A in presence of carbachol and P2Y₂ siRNA

When the tear concentrations of Ap₄A were measured after stimulation with carbachol in the absence and in the presence of P2Y₂ siRNA, it was possible to observe that at 20 and

Fig. 3 Effect of siRNA P2Y₂ in ciliary process. **a** Immunohistochemical localization of P2Y₂ receptors in the rabbit lacrimal gland. **b** Immunohistochemical localization of P2Y₂ after the treatment with the P2Y₂ siRNA as described in methods. **c** Quantification of the fluorescence signal presented in **a** and **b** ($n=4$, *** p <0.001)

Fig. 4 Effect of siRNA P2Y₂ in tear secretion. **a** Time course of siRNA P2Y₂ antagonists in presence of carbachol (10 μ L, 100 μ M) followed by 65 min. Values represent the mean $\pm$ S.E.M. of six independent experiments. *** p <0.001, ** p <0.01, * p <0.05 (two-way ANOVA with Bonferroni's posttest). **b** Effect of siRNA P2Y₂ in tear secretion. siRNA was applied in three consecutive days, and tear secretion was measured during 5 days

35 min, the increase in the dinucleotide concentration was 37 % and 56 %, respectively ($n=8$, Fig. 5a). In order to see whether the increase of the concentration of Ap₄A was due to an increase in its release or if it was an effect produced by the reduction in tear volume, the amount of the dinucleotide was measured for each given time. As observed in Fig. 5b, there were small differences among the amounts of carbachol-

Fig. 5 Effect of siRNA P2Y₂ in the amount of Ap₄A in tear secretion stimulated by carbachol. Time course of Ap₄A levels with siRNA P2Y₂ antagonists in presence of carbachol. Values represent the mean $\pm$ S.E.M. of four independent experiments * p <0.01 (two-way ANOVA with Bonferroni's posttest)

induced Ap₄A either in the presence or in the absence of the P2Y₂ siRNA ($n=8$).

Discussion

The present experimental work describes the involvement of the dinucleotide Ap₄A on tear secretion and how its effect is related by the cholinergic component that regulates tear secretion. Apart from the expected blockade performed by the muscarinic antagonists, the application of P2 receptor antagonists and P2Y₂ siRNA partially blocked the stimulatory effect of the cholinergic agonist carbachol, suggesting some sort of connection between acetylcholine and Ap₄A. Interestingly, there seem to exist a delay in the effect of carbachol when the P2Y₂ antagonist cocktail was assayed. It could be the case that the antagonism of the P2Y receptors is avoiding the phosphorylation of muscarinic receptors that regulate tear secretion, reducing and delaying this physiological process. A similar phenomenon has already been described for M3 muscarinic receptors in the brain, when the lack of phosphorylation produces a clear reduction on acetylcholine activity [27].

The modulation of tear secretion is performed by the autonomic nervous system, where both sympathetic and parasympathetic components, mainly by the transmitters noradrenaline and acetylcholine, rule not only the water production but also mucin secretion [28]. This control is accompanied by the existence of secretagogue compounds present in tears such as diadenosine polyphosphates. Diadenosine polyphosphates and in particular diadenosine tetraphosphate or Ap₄A, can stimulate tear secretion by acting on P2Y₂ receptors present in the lacrimal and accessory glands [29]. The release of this dinucleotide occurs mainly by the ocular surface shear stress as demonstrated in vivo and recently in vitro [25].

Nonetheless, there is a contribution to the nucleotide content that comes from cholinergic and adrenergic nerve endings since they are co-stored with ACh and noradrenaline, being also released after nerve stimulation, as previously demonstrated [30]. Altogether, it is necessary to bear in mind that all the Ap₄A present in tear comes from two main sources, the corneal and conjunctival epithelium and the nerve endings that innervate different ocular structures present in the ocular surface. With this scenario, there is a continuous and regular amount of the dinucleotide since the main source is the ocular surface epithelia due to the blinking process. This dinucleotide stimulates a P2Y₂ receptor that shares the same intracellular second messenger cascade than the one triggered by muscarinic receptors. Therefore, it seems quite obvious that the effect of the P2Y₂ antagonists or siRNA silencing of this receptor blocks the dinucleotide-induced tear secretion and allows seeing the contribution that the cholinergic agent

produces on its own. Since the P2Y₂ receptor is activated by the constant release produced by blinking [24], the tone of Ap₄A may contribute to an increased intracellular level of DAG and IP₃ that may imply that with a small amount of acetylcholine, or in our case carbachol, it may produce a more robust tear production than when the blockade of the P2Y₂ receptor is done. The increased levels of DAG stimulate protein kinase C (PKC), this being the final responsible of the secretory process in the lacrimal gland. In animal models, such as the rat, five known isoforms of PKC are expressed including the canonical PKC, PKC- α , but also PKC- δ , PKC- ϵ , PKC- μ , and PKC- ι/λ . Of all the possible PKCs, only PKC- α has been demonstrated to be translocated by carbachol [31].

Interestingly, cholinergic agonists can also stimulate phospholipase D (PLD) contributing to the lacrimal gland secretory process [32]. In this sense, also, P2Y₂ receptors can trigger this PLD in some cellular models [33]. If this occurs also in the lacrimal gland, this would be another connecting point between cholinergic and purinergic pathways in the regulation of tear secretion.

The connection between cholinergic and purinergic transmissions occurs not only between muscarinic and P2Y metabotropic receptors but also with P2X ionotropic receptors. There are P2X₃ and P2X₇ receptors in the lacrimal gland activated mainly by ATP that increase intracellular concentration of Ca²⁺, triggering protein release. Interestingly, the interaction between muscarinic and P2X₇ receptors is due to the release of ATP from the lacrimal gland. Dartt and Hodges suggest that cholinergic agonists stimulate the release of ATP from efferent nerve endings rather than from the acinar and duct cells [34]. This may explain why higher Ap₄A concentrations are measured when carbachol is applied. Carbachol is facilitating the release of the dinucleotide, presumably from the nerve endings as indicated, and therefore contributing to tear production. Under physiological conditions, the parasympathetic innervation releases acetylcholine as well as Ap₄A in a first step. Secondly, ACh, is able to trigger a secondary pool of Ap₄A by presumably nerve stimulation which is necessary to keep the correct level of hydration on the ocular surface.

An interesting aspect arises when the siRNA for the P2Y₂ receptor is assayed. As observed in Fig. 5, it was possible to measure higher concentrations of the dinucleotide when the siRNA is present. This increase is due to the concomitant reduction in tear volume produced by the oligonucleotide. This is also indicating that the amounts of Ap₄A remain stable when the siRNA for the P2Y₂ is used.

In summary, the combined effect of carbachol and Ap₄A demonstrates that the naturally occurring dinucleotide diadenosine tetraphosphate is, together with ATP, a relevant agonist that firmly contributes to the correct wettability that helps to keep the proper health of the ocular surface.

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