ATP is released into the rabbit eye by antidromic stimulation of the trigeminal nerve

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Antidromic stimulation of the trigeminal nerve produces an irritative response in the rabbit eye characterized by ipsilateral miosis, hyperemia, elevated intracocular pressure, and a disruption of the blood-aqueous barrier. The latter is a bilateral effect. The mediator or mediators involved in this response of the eye are unknown. Increased ATP levels in aqueous humor could be found after trigeminal stimulation. Treatment of rabbits with dipyridamole further increased ATP levels in aqueous humor after stimulation, confirming the findings of Holton that stimulation of sensory nerves causes a release of ATP. Intravitreal injections of ATP could not reproduce the ocular irritative response; however, an iridial hyperemia of long latency and an increase in aqueous humor protein levels were produced. The mechanism of this part of the reaction requires further study.

Key words: adenosine triphosphate, trigeminal nerve, aqueous humor, antidromic stimulation, irritative response, ocular hyperemia, dipyridamole

Antidromic stimulation of the trigeminal nerve produces an irritative response in the rabbit eye characterized by ipsilateral miosis, hyperemia of the iris and conjunctiva, intracocular hypertension, and a disruption of the blood-aqueous barrier. This last effect is also present in the contralateral eye. The irritative response produced by stimulation of this neural pathway suggested that the effects were the consequence of a trans-mitter receptor mechanism. The substance or substances released by trigeminal nerve stimulation have not been identified. The purpose of this study was to learn whether adenosine triphosphate (ATP) is released into the rabbit eye by trigeminal nerve endings after antidromic stimulation and whether ATP could stimulate an irritative response.

Material and methods

Animals. New Zealand, male, albino rabbits weighing 2 to 3 kg were used. When required, general anesthesia was induced with sodium pentobarbital, 15 mg/kg intravenously.

Experimental groups. The trigeminal nerve was exposed and mechanically stimulated as previously described. One, 5, 10, and 20 min after stimulation, the aqueous humor of both eyes was obtained by paracentesis and set on crushed ice. Animals with exposure of the trigeminal nerve but not stimulated served as controls. Aqueous humor of anesthetized unstimulated rabbits and normal rabbits under topical anesthesia was obtained at the same time. ATP concentration was deter-
ATP released by antidromic stimulation

mined in all aqueous humor samples within minutes of finishing the experiment.

Six rabbits were pretreated with 250 μg/kg intravenous dipyridamole 30 minutes before trigeminal stimulation. Three rabbits were treated with intraperitoneal dipyridamole, 1 mg/kg, divided into two doses, administered 3 and 1 hr before the nerve stimulation. (Dipyridamole was kindly supplied by Boehringer Ingelheim, N. Y.) Aqueous humor of these last two groups was obtained by paracentesis 1 min after stimulation and assayed for ATP concentration.

ATP measurement in neural tissue. Normal rabbits were sacrificed with an overdose of sodium pentobarbital. The jugular veins were sectioned and the head of the animal was then washed out of blood with ice-cold saline perfusion through the carotid arteries. The trigeminal nerve and Gasserian ganglion were excised and wet weighed separately. The tissue pieces were mechanically homogenized in ice-cold water, boiled for 10 min, then centrifuged for 15 min at –10° C and 800 x g, in a Sorvall centrifuge. The supernatant was assayed for ATP concentration.

ATP assay. ATP concentration in the test samples was determined with the firefly (Photinus spiralis) luciferin-luciferase system. The samples were boiled for the recovery of ATP because the enzyme is inhibited by a number of substances, including various quenching agents. The light rate was measured with a Packard Tricarb liquid scintillation spectrometer at tritium setting, 65% gain, 3 sec after mixture of the test sample with the crude luciferase preparation. A standard curve for ATP from 2 to 50 pM was obtained with each ATP assay.

In addition, internal standards were used, the test sample was divided into two halves, a known amount of ATP was added to one half before boiling, and the same amount was added to the other half after boiling.

ATP concentration in whole blood was determined in eight rabbits. ATP measurements were also done in aqueous humor of four animals 1 min after disruption of the lens with a needle through the pars plana.

Effect of ATP on the iris preparation. Four irises of normal rabbits were mounted between two plexiglass rings like a diaphragm and set into a 10 ml chamber at 25° C filled with buffered solution according to the method of Farnebo and Hamberger, and oxygen 95% was bubbled into the solution. The pupil was allowed to stabilize for a 30 min period, then ATP was added to reach final concentrations of 10^-8M, 10^-7M, and 10^-6M.

The pupillary diameter was measured with a caliper after addition of ATP to the bath. At the end of the experiment acetylcholine was added to two and norepinephrine to the remaining two preparations, and pupil diameter was again recorded.

Intravitreal administration of nucleotides. Ten microliters of a solution containing 28 nmol ATP, adenosine diphosphate (ADP), or adenosine monophosphate (AMP) were administered to three groups of rabbits. The solution was injected with a 30-gauge needle through the pars plana into the vitreous under topical anesthesia. Ten microliters of saline were injected to the contralateral eye, which served as a control. The animals were observed in a single-blind fashion for a 36 hr period; the pupil diameter and iris conjunctival hyperemia were clinically recorded. Aqueous humor was obtained at 30 min and 5, 18, 24, and 36 hr after drug administration, and protein concentration determined with refractometry of a 50 μl sample. Rabbits after paracentesis were eliminated from the group under observation.

Statistical analysis. The significance of the results was calculated with the t test for sample means or, where specified, with the modified Wilcoxon-White test for samples of different size. All data are given as the mean ± standard error of the mean.

Results

ATP in aqueous humor of normal rabbits. Fourteen samples were assayed: the ATP present was 4.50 ± 0.7(14) nmol/L. There was no significant difference in ATP between paired samples of aqueous humor nor in that of normal aqueous humor obtained from rabbits under topical or general anesthesia.

ATP in aqueous humor of rabbits with unilateral stimulation of the trigeminal nerve. When the trigeminal nerve of one side was stimulated the irritative response previously reported could be observed. ATP concentration in aqueous humor measured 1 min after stimulation was higher than in controls. Interestingly, ATP of the contralateral side was also higher than in controls. The mean for controls was 3.8 ± 0.7 nmoles/L (11). One minute after stimulation the aqueous humor of the stimulated side was 16.2 ± 3 nmol/L (15) in the treated side (p < 0.01) and 14.7 ±
Table I. ATP content of neural tissue

<table>
<thead>
<tr>
<th>Trigeminal nerve (µg/gm.)</th>
<th>Gasserian ganglion (µg/gm.)</th>
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<tr>
<td>37.7</td>
<td>31</td>
</tr>
<tr>
<td>28.5</td>
<td>27.7</td>
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<tr>
<td>37.7</td>
<td>19.4</td>
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Fig. 2. ATP concentration in aqueous humor of stimulated (ST) and of contralateral (CL) side 1 min after stimulation of the trigeminal nerve in animals without treatment (DIP 0) and in animals pretreated with dipyridamole (DIP) 250 and 1000 µg/kg. Mean ± S.E.M.

Effect of ATP, ADP, and AMP on the rabbit eye after intravitreal injection. Thirty minutes after intravitreal injection of ATP no significant change in the clinical aspect of the eye could be observed. The protein contents

ATP in aqueous humor of rabbits pretreated with dipyridamole after unilateral stimulation of the trigeminal nerve. When intravenous dipyridamole, 250 µg/kg, was administered 30 min before stimulation, the ATP level in aqueous humor of the stimulated side was 10.4 ± 5 nmol/L (6), and that of the contralateral side was 8.85 ± 3.7 (6). These measurements were not different from those in the stimulated group that did not receive the drug. When dipyridamole was administered intraperitoneally, 1 mg/kg, ATP 1 min after stimulation was 36.9 ± 12 nmol/L (3) in the stimulated side and 24.8 ± 14 (3) in the contralateral side, values significantly higher than in the stimulated group (Fig. 2) without dipyridamole treatment (p < 0.01, Wilcoxon-White test).

Effect of various ATP doses on the iris diaphragm preparation. ATP had no effect in vitro on the pupil diameter. In four iris preparations ATP concentration reached 10⁻⁴M without producing a change in the pupillary diameter. The preparation responded appropriately to 10⁻⁴M acetylcholine and noradrenaline at the end of the experiment (Fig. 3).
Fig. 4. Protein concentration in aqueous humor (mg/dl) at 30 min and 8, 18, 24, and 36 hr after intravitreal administration of 28 nm of ATP to the treated side (▲) and saline to the control side (●). Mean ± S.E.M.

Fig. 5. Protein concentration in aqueous humor (mg/dl) at 30 min and 8, 18, and 24 hr after intravitreal administration of 28 nmol ADP to the treated side (▲) and saline to the control side (●). Mean ± S.E.M.

of aqueous humor of the treated and untreated sides were 130 ± 48 mg/dl (5) and 130 ± 27 (5), respectively. Eight hours after injection no change could be observed; the protein content of the treated and untreated sides was 50 mg/dl. Eighteen hours after injection a mild hyperemia of the treated iris could be observed and protein measurements revealed 523 ± 9 mg/dl (11) and 66 ± 13 (9), respectively, in the treated and untreated sides (p < 0.01). A trend toward normalization of the disruption of the blood-aqueous barrier could be observed, in that protein in the aqueous humor diminished progressively over the 36 hr period of observation (Fig. 4). The pupil remained unchanged at all times.

After ADP was injected into the vitreous, a similar picture could be observed: no change in pupil diameter, mild hyperemia at 18 hr, and a more pronounced increase in aqueous humor protein concentration (p < 0.01) (Fig. 5).

When AMP was injected into the vitreous, no clinical effect could be detected on the blood-aqueous barrier. Protein measure-
ments 30 min after injection were 162 ± 67 mg/dl (4) and 72.6 ± 36 (4), respectively, in the treated and untreated sides. Eighteen hours after, these measurements were 58 ± 2.6 mg/dl (6) and 60 ± 2.5 (5).

**ATP measurements in blood and in aqueous humor after lens disruption.** ATP in whole rabbit blood was 1.06 ± 0.15 nmol/L (8). These values coincide well with previously reported measurements. ATP in aqueous humor after lens disruption was 61.0 ± 3.4 nmol/L (4) as opposed to the contralateral side, 7.4 ± 2.5 (4) (p < 0.01).

**Discussion**

The effects of trigeminal nerve stimulation on the rabbit eye are well established in the literature. A consensual effect on the blood-aqueous barrier also characterizes the effect of this nerve stimulation. Chemical irritants such as nitrogen mustard have been demonstrated to exert their effect upon the eye by way of the trigeminal nerve in the rabbit.

Dose-response curves of iridial conjunctival hyperemia, miosis, intraocular hypertension, and disruption of the blood-aqueous barrier that follow stimulation of this neural pathway with nitrogen mustard strongly suggest the effects to be the consequence of a transmitter receptor mechanism. The substance or substances released by trigeminal stimulation are not known. Sympathetic denervation does not prevent the effects of trigeminal stimulation nor that of nitrogen mustard applied topically to the eye.

Experiments in the atropinized animal or with degenerated third nerve have shown that these do not eliminate the effects of trigeminal stimulation. Inhibition of prostaglandin synthesis by indomethacin does not prevent the effect of topical nitrogen mustard or trigeminal stimulation. Prostaglandins have not been demonstrated in aqueous humor after nerve stimulation. Analogy between the mechanism of trigeminal irritation and axon reflex skin vasodilatation has been discussed. "Pain" fibers might be involved in both reactions, but the mediator is unknown. Evidence has been presented that vasodilatation in the rabbit ear after antidromic nerve stimulation is mimicked by ATP injections, and it has been shown that ATP is liberated when the great auricular nerve of the rabbit is stimulated.

There is speculation about the possible role of ATP in nervous tissue for the maintenance of membrane permeability and as a cofactor for the vesicular storage of norepinephrine. ATP has been demonstrated to be released from motor nerve terminals during stimulation, and the trophic function of motor fibers has been related to this release. A nonadrenergic inhibitory nerve system has been described in the gut. These fibers release ATP upon stimulation and have been named purinergic nerves. Indirect evidence for the existence of an ATP-releasing nerve fiber system has been presented in the eye. Whether this sort of system might be involved in controls for ordinary levels of intraocular pressure or involved in the irritative ocular response is not known.

Considerable ATP was present in the trigeminal nerve and in the Gasserian ganglion in the present work. It was less than that reported for other nerves. Perhaps the difference might represent losses during the postmortem delay. Nevertheless, ATP measured represents strictly that of neural tissue, since blood contamination was avoided. The purpose of these measurements was to find out whether there was a difference in the ATP content of ganglion and nerve that might allow speculation about the origin of ATP released. The results presented do not allow such determination, and investigation of this part will need to be conducted at a subcellular level. The trigeminal nerve provides fibers for the sensation of the cornea, and it supplies the iris with an important myelinated nerve fiber network. When the nerve is stimulated, a significant increase in the content of ATP in the aqueous humor occurs. Aqueous humor is an extracellular fluid bathing the anterior segment of the eye and is essentially free of cells. After stimulation, cells did not increase significantly and
could not constitute a source of the increased ATP. The increase in ATP content of aqueous humor was bilateral. The crossed fibers of the trigeminal nerve innervating the contralateral eye\textsuperscript{2, 3} are probably the origin of the ATP detected in the contralateral aqueous. ATP in blood is in the red cells; plasma is essentially free of ATP. Thus the disruption of the blood-aqueous barrier that follows trigeminal blood-aqueous barrier that follows trigeminal nerve innervating the contralateral eye\textsuperscript{2, 3} are probably the origin of the ATP detected in the contralateral aqueous. ATP in blood is in the red cells; plasma is essentially free of ATP. Thus the disruption of the blood-aqueous barrier cannot account for the increase observed. Treatment of animals with dipyridamole, 1 mg/kg, further increased ATP levels in aqueous humor after stimulation. Dipyridamole, a coronary vasodilator, inhibits the 3'5'-AMP phosphodiesterase.\textsuperscript{22} It also inhibits ATP uptake from blood by heart and lung\textsuperscript{23} and blocks the reuptake of ATP by purinergic nerve endings.\textsuperscript{18}

The increase in ATP detected after stimulation in animals treated with dipyridamole strengthens the possibility that the actual source of ATP increase is from neural tissue.

When ATP or related nucleotides were injected into the vitreous, the physiopathological effect of trigeminal stimulation could not be mimicked, although an iridial hyperemia of long latency and an increase in aqueous humor protein levels was elicited. Twenty-eight nanomoles injected into the vitreous represent roughly a final concentration of 10\textsuperscript{-5}M. This represents about 10\textsuperscript{5} times the ATP measured in the aqueous humor 1 min after stimulation. Pharmacological matching of the effect observed after nerve stimulation has been a problem in the classic works on neurotransmitter substances.

Holton\textsuperscript{18} reported a discrepancy of 10\textsuperscript{5} between the ATP collected and the ATP needed to match vasodilatation of the great auricular nerve stimulation. Brown et al.\textsuperscript{24} reported a discrepancy of 100 to imitate a twitch in the case of acetylcholine at the neuromuscular junction. Further studies are required relating doses of ATP to the mild but genuine iridial hyperemia found.

From our experiments it cannot be concluded that ATP is responsible for the irritative response to trigeminal nerve stimulation. It is clear, however, that ATP is released into the eye by antidromic stimulation and that an iridial hyperemia and disruption of the blood-aqueous barrier develop after injection of ATP into the vitreous.

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REFERENCES


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