Reports

The effects of theophylline on cyclic adenosine monophosphate metabolism in lymphocytes from open-angle glaucoma patients.

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The effects of theophylline on cyclic-AMP metabolism were evaluated in lymphocytes from individuals tested topically with corticosteroids and from patients with open-angle glaucoma. Lymphocyte cyclic-AMP phosphodiesterase activity, and kinetic constants were similar in all groups. Theophylline inhibited the enzyme in all groups at similar concentrations and caused an equal rise in cyclic-AMP levels in lymphocytes from each group. It is concluded that the differential effects of theophylline on inhibition of phytohemagglutinin-stimulated lymphocyte transformation in the tested groups cannot be explained by its effect on cyclic-AMP metabolism.

Lymphocytes from patients with open-angle glaucoma (OAG) and from topical corticosteroid responders (GG) are more sensitive to theophylline than those from steroid nonresponders (NN).[1] Lower concentrations of theophylline are required to inhibit phytohemagglutinin (PHA)-stimulated lymphocyte transformation in the OAG and GG groups. Theophylline raises intracellular levels of cyclic 3'5'-adenosine monophosphate (cAMP) by competitive inhibition of cyclic nucleotide phosphodiesterase, the enzyme that metabolizes cAMP to inactive 5'-AMP.[2] Since raised levels of cAMP are associated with decreased lymphocyte transformation, our findings suggested that an abnormality in cAMP metabolism at the level of phosphodiesterase might be involved in the mechanism of increased cellular sensitivity to theophylline.[3]

We studied the effects of theophylline on cAMP metabolism in lymphocytes from populations tested topically with corticosteroids and from patients with OAG. We also isolated and characterized lymphocyte cAMP phosphodiesterase and studied its inhibition by theophylline.

Methods. Subjects. Individuals were selected on the basis of their intraocular pressure response to topical dexamethasone.* Those taking systemic medications known to affect cyclic AMP metabolism, such as estrogen preparations, corticosteroids, or methyl xanthines, were excluded. Venous blood was obtained between 8:00 and 10:00 A.M., after an eight-hour fast, and assays were performed promptly.

Cyclic AMP assay. Cyclic AMP levels in lymphocytes purified by Ficoll Hypaque density gradient centrifugation were measured by Dr. Charles Parker using a radioimmunoassay.[4] Duplicate assays were performed on cell suspensions containing 10^6 lymphocytes, ten and sixty minutes after incubation with theophylline (4.55×10^{-4} M). The average of controls taken at zero time and after incubation without theophylline served as baseline values. The assay is sensititve from 1.0 to 20 picomoles of cAMP and shows no interferences from other cyclic, mono- , di- , and trinucleotides. Results are expressed in picomoles of cAMP per 10^7 lymphocytes.

Phosphodiesterase assay. Purified lymphocytes were prepared as above and washed twice with buffer containing 50 mM Tris, 5 mM MgCl, 2 mM Dithiothreitol, and 0.15 M NaCl, pH = 8.0. Cells were sonicated at a concentration of 50×10^6 lymphocytes per milliliter in buffer without saline and cytosol prepared by centrifugation at 16,000 g for 20 minutes at 4° C. Protein concentration was determined by the method of Lowry and co-workers.[5]

Phosphodiesterase activity was measured using the two-step technique of Butcher and Sutherland[2] as modified by D'Armiente, Johnson, and Pasten.[6] Snake venom *(Ohiophagus Hannah)* was used as a source of 5'-nucleotidase to convert the reaction product (5'-AMP) to adenosine and phosphate. Anion exchange resin (BioRad AG 1-X2, 200-400 mesh, Cl- form) was used to separate unreacted cyclic-AMP from the adenosine.

Each duplicate reaction mixture contained buffer and cytosol at a final volume of 100 μl in 10 by 75 mm. glass tubes. The reaction was initiated by adding 10 μl of unlabeled cyclic AMP (0.25 to 40.0 μM) and 10 μl of ^{3}H-cyclic AMP (50,000 c.p.m., 33.5 mCi per millimole). After incubation at 37° C. for ten minutes, the reaction was stopped by boiling for one minute. Fifty microliters of snake venom (1 mg. per milliliter) was added and the mixture incubated at 37° C. for ten minutes. This reaction was terminated by boiling for one minute, and 1.0 ml. of a continuously stirred 1:3 slurry of resin was added. The mixture was vortexed briefly and centrifuged for 10 mintues at 4,000 g. The supernatant was aspirated into Bray's solution

*Patients are classified as GG if their intraocular pressure rises above 31 mm. Hg after using 0.1 per cent dexamethasone four times a day for 6 weeks or NN if the pressure remains less than 20 mm. Hg.

Table I. Rise in lymphocyte cyclic-AMP in response to theophylline (theophylline concentration 4.55×10^{-4} M; picomoles cAMP per 10^7 lymphocytes)

NN			*GG*			*OAG*		
Age	*10 min.*	*60 min.*	*Age*	*10 min.*	*60 min.*	*Age*	*10 min.*	*60 min.*
29	4.7	3.8	27	4.7	2.0	49	−0.6	1.2
29	5.4	1.0	28	3.0	−0.2	54	4.7	0.8
42	0.0	0.6	67	0.9	1.0	60	2.3	0.6
45	1.0	0.4	71	1.3	2.5	62	2.5	1.4
49	1.1	1.9				67	0.0	−2.0
70	1.7	0.4		—	—	71	2.5	1.8
Mean	2.3	1.3		2.5	1.3		1.9	0.6
S.E.M.	0.9	0.5		1.7	1.2		0.8	0.5

Table II. Lymphocyte phosphodiesterase activity (picomoles per milligram of protein minute at substrate concentration 1.0 μM, mean and standard error)

	No.	*Activity*
NN	7	48.9 ± 4.3
GG	7	47.1 ± 4.9
OAG	7	56.7 ± 4.5

and counted in a Packard liquid scintillation counter. The blank for each experiment was a mixture inactivated immediately by boiling. Activity is expressed in picomoles of cyclic-AMP per milligram of protein per minute. The assay is linear with time for 20 minutes and with protein concentration up to 50 μg.

Results. Cyclic AMP levels. The baseline levels of lymphocyte cAMP during incubation with buffer for 10 and 60 minutes were the same for the NN, GG, and OAG groups. The values at 10 minutes were between 3.3 and 4.5 pmoles of cAMP per 10^7 lymphocytes. At 60 minutes, the average values were between 2.0 and 3.6 pmoles of cAMP per 10^7 lymphocytes.

Table I gives the net rise in cAMP after incubation for 10 and 60 minutes. The concentration of theophylline is similar to that used in the lymphocyte transformation studies, and the 60-minute values correspond to the rise in cAMP which would be expected at the start of PHA stimulation in our lymphocyte transformation studies.[1] The cAMP response to theophylline is greater at 10 than at 60 minutes. There are no significant differences in the cAMP responses at 10 and 60 minutes among the three groups.

Phosphodiesterase activity. Table II gives the cAMP phosphodiesterase activity in 21 subjects classified according to topical corticosteroid response. The activity of the enzyme is similar to values previously reported.[7] The activity of the enzyme in the three corticosteroid-tested groups is similar, and there was no correlation with age. Table III gives the kinetic constants of the enzyme, Km and V_{max}, determined by regression analysis of Linweaver-Burke plots. The two sets of kinetic constants are consistent with findings in other tissues suggesting at least two forms of the enzyme.[8] Statistical analysis of these kinetic constants shows no significant differences among the groups, except for the difference between NN and GG for the high Km ($p < 0.025$). In all other respects, the enzymes are kinetically identical.

Table IV shows the concentration of theophylline required to half-inhibit cAMP phosphodiesterase activity at a substrate concentration of 1.0 μM. Since theophylline inhibition was studied at a substrate concentration near the Km for the enzyme, and theophylline is a simple competitive inhibitor, the concentration of theophylline required to half-inhibit the enzyme has a simple relationship to Ki and accurately reflects the sensitivity or binding of theophylline to the enzyme. Statistical analysis shows no significant differences among the three groups.

This table also compares the concentrations of theophylline required to inhibit lymphocyte transformation by 50 per cent with the concentration required to inhibit the enzyme in vitro. The concentrations required to inhibit the enzyme are 10 per cent higher for the NN group and 50 per cent higher for the GG and OAG group.

The theophylline concentration required to half inhibit the high Km phosphodiesterase was 6 mM or 15 to 25 times that required to inhibit lymphocyte transformation. Therefore, at the theophylline concentrations that inhibit lymphocyte transformation, the high Km enzyme is unaffected.

Discussion. Drugs, such as theophylline, which raise intracellular levels of cAMP are potent inhibitors of lymphocyte transformation[3] and other lymphocyte functions such as mediator release, antibody synthesis, and cytotoxicity. Our previous

Table III. Lymphocyte phosphodiesterase kinetics (Km in μM cAMP; V_{MAX} in picomoles of cAMP per milligram of protein per minute: mean and S.E.)

		Low		*High*	
	No.	*Km*	V_{max}	*Km*	V_{max}
NN	6	2.38 ± 0.51	157 ± 24	10.59 ± 0.99	298 ± 35
GG	7	1.70 ± 0.23	130 ± 19	6.13 ± 0.68	233 ± 39
OAG	5	1.77 ± 0.28	162 ± 24	9.44 ± 1.26	300 ± 40

Table IV. Comparison of theophylline concentration required to inhibit lymphocyte transformation and phosphodiesterase activity 50 per cent (× 10^{-5} M, ± S.E.M.)

	NN	*GG*	*OAG*
Lymphocyte transformation	39.8 ± 1.6 (10)	26.4 ± 1.6 (10)	24.3 ± 1.8 (7)
Phosphodiesterase activity	44.9 ± 6.0 (7)	38.1 ± 3.2 (9)	38.4 ± 5.6 (8)

study[1] demonstrated that lower concentrations of theophylline inhibit transformation in lymphocytes from glaucoma patients compared to normal subjects. Since the concentration of theophylline that inhibits lymphocyte transformation is approximately the same as that required to inhibit cAMP phosphodiesterase in vitro, its differential effect on lymphocyte transformation in the glaucomatous population was thought to be due to its effects on lymphocyte cAMP metabolism.

We found that lymphocyte cAMP phosphodiesterase has similar activity and kinetic constants in the glaucomatous population and in nonresponders to topical corticosteroids. Furthermore, the concentration of theophylline required to inhibit the isolated enzyme is similar for all groups. This lack of differential effect of theophylline on the isolated enzyme did not rule out a differential effect on whole lymphocytes. However, equal rises in cellular cAMP levels occurred when lymphocytes from all groups were incubated with theophylline, proving that at the concentrations used to inhibit lymphocyte transformation, theophylline has similar effects on cAMP metabolism in intact lymphocytes from all groups. Therefore, the differential effects of theophylline on lymphocyte transformation in the glaucomatous population must involve some other aspect of cellular transport or metabolism. This is not unreasonable since other agents such as ouabain, diphenylhydantoin, aspirin, and corticosteroids inhibit transformation without affecting cAMP metabolism.[9, 10]

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The effects of elevated intraocular pressure on slow axonal protein flow.*

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The effect of an elevation of the intraocular pressure on the slowly moving axonal protein flow within the retinal ganglion cells was studied in six rhesus monkeys. Unilateral elevation of the intraocular pressure was obtained by an intracameral injection of alpha-chymotrypsin. There was a marked initial reduction of slow axonal flow into the optic nerve of the affected eye, followed by a recovery of flow as intraocular pressure returned toward normal. Reduction in slow axonal flow was noted even with minor elevations in intraocular pressure. A portion of this obstructed flow appeared to accumulate within the retina. It has not yet been determined whether this inverse relationship between intraocular pressure elevation and slow axonal protein flow relates to pressure, ischemia, or both effects upon the retinal ganglion cell axons.

The discovery of the axonal transport of various soluble proteins, molecules, and subcellular particles within the optic nerve has aroused interest in the functional significance of this process[1] and its possible relationship to ischemia in glaucoma and other diseases.[2] Microtubules and neurofilaments, some mitochondria, and much of the soluble intracellular protein move down the axon at a rate of 1 to 2 mm. per 24 hours and is referred to as slow axonal flow.[3] These substances are the constantly renewing, structural components of the axon. The purpose of this study was to characterize the slow component of axonal protein flow under conditions of elevated intraocular pressure and to determine if a recovery of slow flow occurred following the return of the intraocular pressure toward normal levels.

Materials and methods. Six, three kilogram, macacus rhesus monkeys were used in this study. Each animal had an ocular examination, including applanation tonometry and funduscopic photography. Fluorescein angiography of the ocular fundus and discs was performed to determine the pattern and adequacy of blood flow to the posterior segment and optic nerve of each eye. Baseline electroretinographic signals were obtained in each eye and were normal in all cases. Intraocular pressure was measured by MacKay-Marg tonometry on three occasions under ketamine hydrochloride anesthesia prior to producing an elevation of intraocular pressure in one eye.

Intraocular pressure was altered by the injection of 175 units of alpha-chymotrypsin into the posterior chamber of the right eye as described by Zimmerman, de Vencie, and Hamasaki.[4] Saline was injected into the left eye of these animals and into both eyes of a control animal. Intraocular pressures were recorded daily following the injections.

In most instances, elevation of the intraocular pressure was associated with subluxation of the lens, dilation of the pupil, and corneal edema. Indirect ophthalmoscopy was possible and the discs and retina appeared normal.

Four days after injection of the alpha-chymotrypsin, 20 lambda of tritiated leucine (1 Ci. per liter) were injected intravitreally into both eyes of three animals on three successive days. Another monkey, who did not receive alpha-chymotrypsin in either eye, was otherwise treated identically. These animals had repeat electroretinographic measurements on the morning of the eighth day prior to death.

In two additional monkeys, an anterior chamber paracentesis was performed in each eye and 0.2 c.c. of aqueous removed. A single injection of 200 lambda of tritiated leucine was placed intravitreally into each eye. Twenty-four hours later the pressure was elevated by injecting 175 units of alpha-chymotrypsin in the posterior chamber of the right eye only, while saline was injected into the left. Intraocular pressures were measured daily and the animals killed four days after the injection of the alpha-chymotrypsin.

The monkeys were deeply anesthetized with pentobarbital and ketamine, anticoagulated with heparin, and then perfused through the aorta with a 10 per cent buffered formaldehyde solution. The eye, optic nerve, and brain were promptly removed.

After the tissues were removed, a needle with 10-0 monofilament nylon suture was placed through the superficial glia of the optic nerve at twelve o'clock where it entered the globe. The nerve was carefully excised from the eye with the optic nerve head remaining within the globe. The nerve was cut centrally just before it entered the chiasm.

The nerve was washed and then dehydrated in ascending concentrations of alcohol. Alcohol was replaced by paraffin, maintaining orientation of the tissues.

The resultant block was trimmed and eight micra sections were serially cut. Ten serial sections were pooled for scintillation counting and protein determination. The tissue was digested in 1.0 M sodium hydroxide. Protein determinations were performed according to the method of Lowry and co-workers.[5] Adjacent sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), and toluidine blue. A matched