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Receptor-responses in fresh human ciliary muscle

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1 The physiological and pharmacological properties of ciliary muscle isolated from fresh human eyes were investigated.

2 The muscle exhibited no spontaneous activity. Concentration-dependent contractions in response to carbachol were competitively antagonized by atropine ($pA_2 = 8.95$).

3 The muscle, precontracted by carbachol (2.7×10^{-4} M), responded to the application of isoprenaline by concentration-dependent relaxation blocked by propranolol (3.5×10^{-9} M to 3.5×10^{-8} M; $pA_2 = 9.15$).

4 Angiotensin-evoked contractions were antagonized by 8-Ala-angiotensin II (4.5×10^{-8} M) in a competitive manner, but were not inhibited by phentolamine or propranolol.

5 Contractions generated by electrical stimulation of the muscle (30 ms, 20 Hz, 60 pulses) were antagonized by atropine (10^{-7} M) and tetrodotoxin (6.3×10^{-7} M). Phentolamine and propranolol did not influence these responses.

6 An increase of the external potassium concentration ($[K^+]_o$) from 5.4 to 158.8 mM produced a mechanical response, antagonized by atropine, but not influenced by tetrodotoxin, phentolamine or propranolol.

7 The human ciliary muscle appears to carry muscarinic and angiotensin receptors and β_2-adrenoceptors. The estimate of $K_{atropine}$ for muscarinic receptors mediating carbachol-induced contractions agrees with estimates of $K_{atropine}$ reported for human and rabbit iris.

Introduction

Many authors have carried out research on intraocular samples of animals in order to analyse the receptor-responses and their localization in the eye. However relatively few papers have been written on human tissue and nearly always refrigerated eyes have been used since fresh human material is hard to obtain. Van Alphen (1976) analysed the distribution of adrenoceptors in human muscle strips dissected from eyebank eyes, refrigerated at 4°C. More recently Kaumann & Hennekes (1979) investigated the affinity of atropine for muscarinic receptors in human fresh sphincter pupillae. Previously we analysed the receptor distribution of fresh human iridial muscle in both the radial and circular fibres (Reibaldi *et al.*, 1984).

In the present experiments we have investigated the receptor-distribution and the affinity of atropine and propranolol respectively for muscarinic receptors and β_2-adrenoceptors in fresh human ciliary muscle. In addition, the effects of atropine and tetrodotoxin on contractions induced by high K^+ or evoked by electrical stimulation with long pulses were investigated and compared with the results of Suzuki (1983) who analysed the pharmacological and physiological characteristics of bovine ciliary muscle. He found that the contractile activity of the ciliary muscle in response to electrical stimulation or high K^+ concentrations was solely dependent on cholinergic nerves and not on the depolarization of the muscle cell *per se*.

The present results suggest that the human ciliary muscle is characterized by an excitability of the muscle cell membrane although the response is also controlled by acetylcholine release from cholinergic nerve terminals.

The atropine-resistant component of the contraction in response to electrical or K^+-stimulation could be attributed to the release of an excitatory transmitter from non-adrenergic, non-cholinergic nerves.

Methods

Eyes from 10 patients, who had to undergo enucleation for different reasons (8 with malignant choroidal melanomas and two with blow-out bulbes), were

immediately immersed in an oxygenated modified Krebs solution. Eyes that had been treated with autonomic drugs because of absolute glaucoma were not used. Ciliary muscle strips were prepared under a binocular microscope. In dissecting the ciliary body from the scleral spur, lens and choroid, great care was taken to avoid damaging the tissue. Sutures were tied to both ends. In the meridional strip one end of the muscle was left adherent to the scleral spur and a suture was passed through a small piece of limbus. Another suture was tied to the choroid at the equator. To investigate the mechanical properties, the strips were set up under a tension of 150 mg in a 20 ml organ bath through which Krebs solution at 37°C flowed continuously and which was aerated with 95% O_2 and 5% CO_2. Contractile responses were measured isometrically by means of a force displacement transducer (Dyo, U. Basile), and recorded on a linear recorder coupled to a Grass polygraph. The stimulating electrodes, 1 mm in width and 6 mm in length, were placed in parallel with the muscle strip. The stimulations were applied supramaximally with an electronic square wave stimulator (Palmer).

The tissues were allowed to equilibrate for 60 min, before any drug addition. Antagonists were placed in contact with the tissues 60 min before agonists. Where more than one concentration of antagonist was added to a tissue, a control response to the agonist was taken before the second concentration of antagonist was added, in order to ensure that complete recovery from blockade had taken place.

Except where otherwise indicated, contractile responses are expressed as a percentage of the maximal effect produced by carbachol before drug treatment; whereas relaxations are expressed as a percentage of the maximal effect produced by isoprenaline.

Differences were examined by Student's t test for paired observations and were considered to be significant for $P < 0.05$.

Apparent pA_2 values for the antagonists were calculated from Arunlakshana & Schild (1959) plots. Dose-ratios were calculated at the EC_{50} level.

$pK_{antagonist}$ values were estimated from the equation

$$K_{antagonist} = \frac{[antagonist]}{dose\text{-}atio - 1}$$

for each concentration of antagonist tested.

Krebs solution was of the following composition (mM): NaCl 136.8, KCl 5.4, $CaCl_2$ 2.7, $MgSO_4.7H_2O$ 0.8, $NaH_2PO_4.H_2O$ 1.4, $NaHCO_3$ 12, glucose 5 and Na-ascorbate 0.2.

The high K^+ solution was prepared by replacing NaCl and $NaHCO_3$ with equimolar amounts of KCl and $KHCO_3$ respectively.

The following drugs were used: carbamylcholine chloride (Sigma), atropine sulphate (Merck), isoprenaline hydrochloride (Fluka), propranolol hydrochloride (I.C.I.L.), phenylephrine hydrochloride (Winthrop), phentolamine hydrochloride (Ciba), angiotensin II (Hipertensin, Ciba), 8-Ala-angiotensin II (Chemicals), prostaglandin $F_{2\alpha}$ (Upjohn), noradrenaline (Sigma), physostigmine (Sigma), neostigmine (Sigma), tetrodotoxin (Sigma), histamine hydrochloride (Fluka), 5-hydroxytryptamine (Sigma), practolol (I.C.I.L.) and atenolol (I.C.I.L.).

Results

After pre-incubation for 1 h, the preparations of human ciliary muscle relaxed and reached a stable tone with a final length of 5–6 mm. Spontaneous activity was absent throughout.

The muscle responded to carbachol by concentration-dependent contractions; the mechanical threshold in terms of carbachol concentration was about 2×10^{-6} M and the maximal contraction was obtained with 2.7×10^{-4} M carbachol. Atropine antagonized the effect of carbachol (Figure 1), the concentration-effect curves being shifted to the right in a nearly parallel and surmountable manner, indicating competitive antagonism (Figure 2a). The threshold concentrations of carbachol shifted to 1.5×10^{-5} M and 4.7×10^{-4} M with 10^{-8} M and 10^{-7} M atropine respectively.

The dose-ratios of carbachol, when plotted as a function of atropine concentration according to Arunlakshana & Schild (1959), resulted in a line of slope 1.1 ± 0.05 (Figure 2b) from which a pA_2 value for atropine (8.95) was calculated. The slope of the Schild plot is close to the slope of unity expected for simple competitive antagonism.

Ciliary muscle strips did not respond to catecholamines unless they had been precontracted by carbachol. After carbachol, 2.7×10^{-4} M, catecholamines induced a relaxation. Isoprenaline was the most potent agonist tested, with a threshold at about 10^{-5} M and a maximum at 7×10^{-4} M. The effect of isoprenaline was completely and readily reversible. Propranolol antagonized the isoprenaline-induced relaxation (Figure 3): concentration-effect curves were shifted to the right in parallel and surmountable fashion (Figure 4a). Analysis of the data by the method of Arunlakshana & Schild (1959) gave a line with a slope which did not differ significantly from unity (1.1 ± 0.03) (Figure 4b) and from which a pA_2 value for propranolol of 9.15 was estimated.

The apparent dissociation constant for the β_2-adrenoceptor-propranolol complex ($K_{propranolol}$) was also estimated giving a value of 7×10^{-10} M. β_1-selective antagonists, practolol and atenolol were ineffective in antagonizing the effect of isoprenaline, indicating that only β_2-adrenoceptors are present in the human ciliary

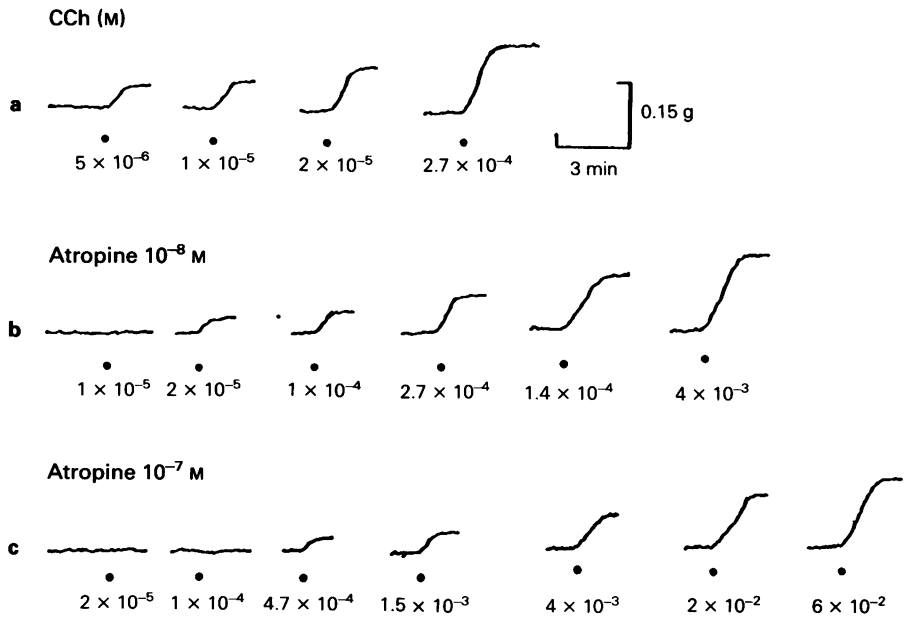

Figure 1 Carbachol (CCh)-induced contractions in a human ciliary muscle (a) in the absence and in the presence of (b) 10^{-8}M and (c) 10^{-7}M atropine. After washing out carbachol, further washes were performed with solution containing the indicated concentration of atropine every 5 min for 60 min so that the tissue was in contact with atropine for 60 min before a dose-response curve for carbachol was determined.

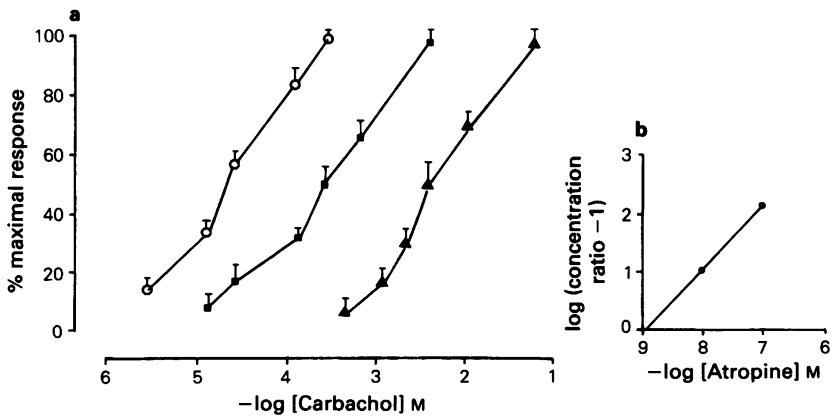

Figure 2 Competitive antagonism by atropine of carbachol-induced contractions in human ciliary muscle. (a) Concentration-effect curves in the absence (○) and in the presence of 10^{-8}M (■) and 10^{-7}M (▲) atropine. Each point represents the mean value with standard error of 7–8 experiments ($P < 0.01$). (b) Schild plot for atropine with carbachol as agonist and 60 min contact time with atropine. The points represent values from 7 experiments.

muscle. Phentolamine (10^{-6} M to 10^{-5} M) had no effect and neither enhanced nor inhibited the relaxation induced by catecholamines. Moreover after β_2-receptor blockade by propranolol (3.5×10^{-8} M), phenylephrine (10^{-5} to 10^{-3} M) did not cause contractions. These results indicate the absence of α-adrenoceptors.

The preparations of human ciliary muscle contracted in response to angiotensin II (Figure 5) with a threshold at about 10^{-6} M and a maximum at 2×10^{-5} M. The effect of 2×10^{-5} M angiotensin was never more than 65–70% of the maximal response to carbachol and was not inhibited by phentolamine (10^{-5} M) or atropine (10^{-8} M to 10^{-7} M) whereas it was blocked by 8-Ala-angiotensin II, 4.5×10^{-8} M, a potent antagonist of angiotensin II (Turker *et al.*, 1971). The nature of the antagonism appears to be competitive, the evidence to support this assumption being: the shift in the curves is parallel and the height of the maximal response to angiotensin is not depressed (Arunlakshana & Schild, 1959) (Figure 5). The effect of 8-Ala-angiotensin II was completely reversible.

Some preparations responded to high concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ 5×10^{-5} M to 10^{-3} M) by contractions that were never more than 20–25% of the maximal response to carbachol. Histamine and 5-hydroxytryptamine did not evoke any response in the human ciliary muscle.

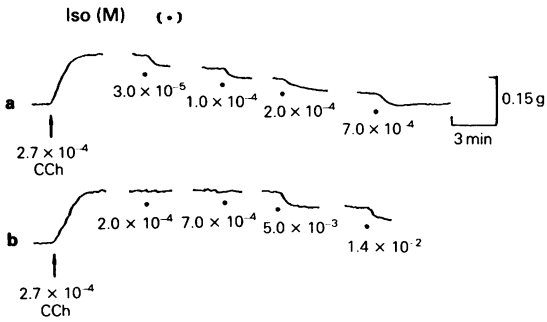

Figure 3 Isoprenaline (Iso)-induced relaxations in a human ciliary muscle precontracted by carbachol 2.7×10^{-4}M (a) in the absence and (b) in the presence of 3.5×10^{-8}M propranolol. After washing out isoprenaline and in the continuous presence of carbachol (2.7×10^{-4}M) further washes were performed with solutions containing the indicated concentration of propranolol every 5 min for 60 min before a dose-response curve for isoprenaline was determined.

Mechanical responses of the ciliary muscle evoked by electrical stimulation

Effects of direct stimulation of the ciliary muscle were investigated using long electrical pulses (20 ms–100 ms) with variable frequencies, conditions that are generally considered to be sufficient to evoke muscle excitation.

The mechanical responses evoked by electrical stimulation (30 ms, 20 Hz, 60 pulses) were about 85–90% of the maximal effect elicited by carbachol (2.7×10^{-4} M) (Figure 6). Atropine (10^{-9} to 10^{-6} M) produced a concentration-dependent reduction in the

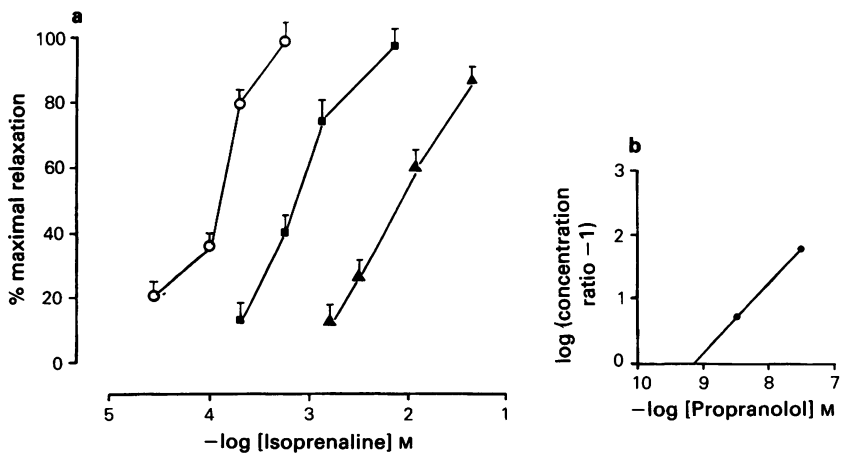

Figure 4 Competitive antagonism by propranolol of isoprenaline-induced relaxation in the human ciliary muscle. The muscle had been precontracted by 2.7×10^{-4}M carbachol. (a) Concentration-effect curves in the absence (○) and in the presence of 3.5×10^{-9}M (■) and 3.5×10^{-8}M (▲) propranolol. Each point represents the mean value with standard error from 8 experiments. ($P < 0.01$). (b) Schild plot for propranolol with isoprenaline as agonist and 60 min contact time with propranolol. The points represent mean values from 8 experiments.

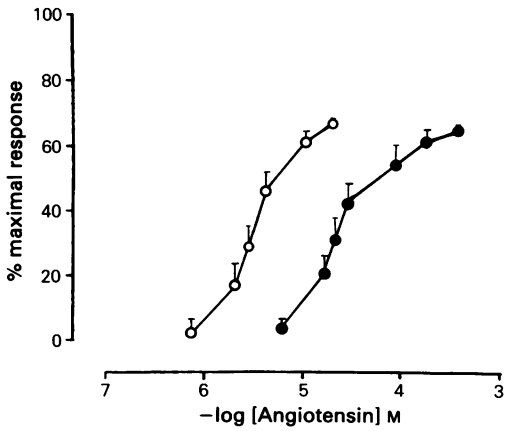

Figure 5 Effect of 8-Ala-angiotensin II on angiotensin II-induced contractions in the human ciliary muscle. Concentration-effect curves in the absence (○) and in the presence (●) of 4.5×10^{-8}M 8-Ala-angiotensin II. On the ordinate scale, contraction is expressed as a percentage of the maximal response to carbachol. Each point represents the mean value with standard error from 9 experiments. Paired Student's t test, $P < 0.01$.

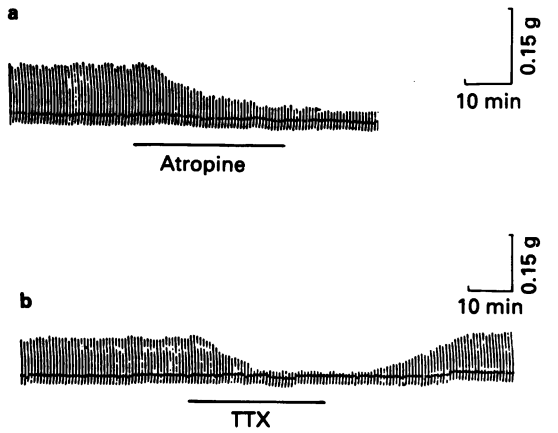

Figure 6 The effect of atropine and tetrodotoxin on the mechanical responses of the human ciliary muscle to field stimulation (30 ms, 20 Hz, 60 pulses). (a) Inhibition by atropine 10^{-7}M. A partial recovery of contractions appeared about 2 h after a washing. (b) Inhibition by tetrodotoxin (TTX) 6.3×10^{-7}M and recovery after washing.

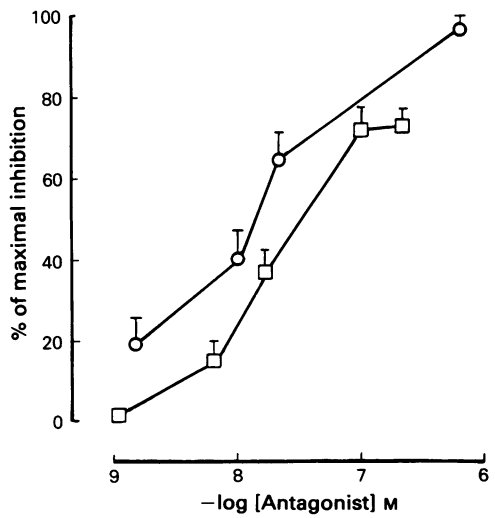

Figure 7 The effect of tetrodotoxin (○) and atropine (□) on the mechanical responses of the human ciliary muscle to field stimulation (30 ms, 20 Hz). Each point represents the mean of values from 4–5 determinations with s.e.mean shown by vertical lines. Only one concentration of antagonist was used in each experiment.

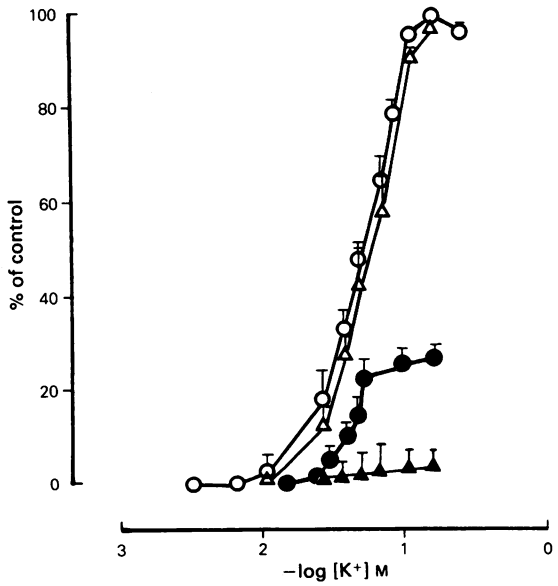

Figure 8 The effect of increasing concentrations of $[K^+]_o$ on the mechanical responses of the human ciliary muscle in the absence (open symbols) and in the presence (closed symbols) of atropine 10^{-7}M. Phasic contractions (○,●); tonic contractions (△,▲). The results were normalized by taking the contractions to 150 mM K^+ without drugs as 100%. Vertical bars show s.e.mean ($n = 10$; $P < 0.01$).

twitch response of the ciliary muscle, but did not abolish it: the maximum inhibition obtained with 10^{-7} M atropine (a concentration sufficient to shift the concentration-response curve to carbachol two log units to the right) was 70 to 75% (Figure 7). The effect of atropine was partially and very slowly reversible (after 2–3 h). Tetrodotoxin (6.3×10^{-7} M) almost completely blocked the development of the evoked tension (Figure 7). Propranolol (3.5×10^{-9} M to 3.5×10^{-8} M) and phentolamine (10^{-6} M to 10^{-5} M) did not influence the amplitude of the evoked contractions.

Effects of external K^+ concentration

Contractions were also produced when the external K^+ concentration ($[K^+]_o$) was increased. There was an initial phasic component following by a tonic component. The amplitudes of the phasic and tonic contraction were plotted against the K^+ concentration as shown in Figure 8. The mechanical threshold in terms of K^+ concentration was about 10 mM and the maximal contraction was reached with 150 mM $[K^+]_o$. Atropine 10^{-7} M caused an inhibition of the phasic and tonic components of the contractile response induced by K^+: the threshold concentration of $[K^+]_o$ shifted to 35–40 mM. Significant differences were observed between the inhibition of the phasic and tonic responses ($P < 0.05$): the maximum phasic contraction was reduced to about 25–30% of the control, whereas the tonic component was almost completely abolished. Tetrodotoxin (6.3×10^{-7} M to 1.2×10^{-5} M), propranolol (3.5×10^{-8} M) and phentolamine (10^{-5} M) did not produce signficant antagonism of the contraction.

Discussion

Ciliary muscle is classified as a multi-unit smooth muscle that is densely innervated. It is generally considered to show no spontaneous activity.

The present experiments have shown that the human ciliary muscle carries β_2-adrenoceptors, muscarinic receptors and angiotensin receptors. The muscle responded to carbachol by concentration-dependent contractions that were antagonized by atropine in a competitive manner. Our estimate of $K_{atropine}$ for muscarinic receptors mediating carbachol-induced contractions in human ciliary muscle agrees with estimates of $K_{atropine}$ reported for the human and rabbit iris by Kauman *et al.* (1979) and Smith (1976) respectively. Furthermore the value of $K_{atropine}$ for the human ciliary muscle also agrees with estimates of $K_{atropine}$ for muscarinic receptors of ileum (Arunlakshana & Schild, 1959; Paton & Rang, 1965) and heart of guinea-pig (Thron & Waud, 1968).

The human ciliary muscle responded to catecholamines when it was precontracted by carbachol. Isoprenaline was the most potent agonist and induced a concentration-dependent relaxation antagonized by propranolol. The fact that β_1-selective antagonists, practolol and atenolol were ineffective in antagonizing the isoprenaline-induced relaxation, suggests that only β_2-adrenoceptors are present in the human ciliary muscle. *In vivo* and *in vitro* studies with selective and nonselective β-adrenoceptor agonists and antagonists are in agreement with this view (Rowland & Potter, 1979; Nathanson, 1980). Phentolamine had no effect and it neither enhanced nor inhibited the relaxations induced by catecholamines. After β_2-receptor blockade by propranolol, phenylephrine, a specific α-adrenoceptor stimulant, did not cause contractile responses indicating the absence of α-adrenoceptors.

The human ciliary muscle also carries angiotensin receptors, since angiotensin II produced concentration-dependent contractions antagonized by the competitive antagonist 8-Ala-angiotensin II, but not by phentolamine or atropine.

The present experiments have also shown that human ciliary muscle contractions evoked by electrical stimulation with long pulses (20 ms–100 ms) were partially inhibited by atropine. These findings suggest that the mechanical activity of the muscle induced by electrical stimulation is only partly due to acetylcholine release from cholinergic nerves. The atropine-resistant component of contraction could be attributed to the release of a neurotransmitter different from those tested in the experiment. This view is also supported by the fact that tetrodotoxin almost completely blocked the contractions evoked by electrical stimulation.

Contractions induced by high K^+ were not influenced by tetrodotoxin, but they were antagonized by atropine (10^{-7} M atropine reduced the maximal phasic contraction to 25–30% of the control). It is well known that tetrodotoxin suppresses nerve activity without affecting the smooth muscle (Kuriyama *et al.*, 1967), however it does not block acetylcholine release via depolarization of cholinergic nerve terminals, in the presence of external calcium (Katz & Miledi, 1966). These findings support the conclusions drawn from the electrical stimulation experiments.

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