Br. J. Pharmac. (1983), **78**, 591–597

Neuronal influence on the mechanical activity of the ciliary muscle

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1 Neuronal effects and the pharmacological properties of the bovine ciliary muscle were investigated *in vitro*. The bovine ciliary muscle exhibited no spontaneous activity.

2 Electrical stimulation of an isolated short ciliary nerve produced distinct contractions. The minimal stimulus duration required to evoke a contraction was 0.2 ms and amplitude of the contraction was maximal at 2 ms. Twitch or incomplete tetanus reached a complete tetanus with 4 Hz stimulation.

3 Raising the external potassium concentration from 5.9 to 158.8 mM produced a contracture which consisted of an initial phasic and then tonic components.

4 The contractions generated by either electrical stimulation (0.2–100 ms) or high K were potentiated by physostigmine and completely inhibited by atropine. Neither adrenoceptor agonists nor blockers influenced these contractions.

5 Application of tetraethylammonium (TEA), potentiated the electrically-induced ciliary muscle contraction, and the effect of TEA was not completely inhibited by high concentrations of either atropine or tetrodotoxin. Thus, TEA presumably acts both pre-junctionally and post-junctionally to increase the contractile development of ciliary muscle.

6 The ciliary contractile response is primarily mediated by acetylcholine released from nerves, and this response is accompanied by a negligible contribution from the sympathetic nerves. Depolarization induced by electrical currents or by high K was ineffective in evoking contraction of the ciliary muscle.

7 The results suggest that excitation of the ciliary muscle is probably mediated via junction potentials or by a direct transmitter action without any very great change in the potential. Action potentials are probably generated in the presence of TEA.

Introduction

Ciliary muscle is classified as a multi-unit smooth muscle which is densely innervated (Ruskell & Griffiths, 1979) and it is generally considered to show no spontaneous activity. Although the electrical and mechanical properties of smooth muscle have been widely described (see, for example, Bolton, 1979; Bülbring, Brading, Jones & Tomita, 1981), attention has not been given to the neuronal effects or the physiological properties of the ciliary muscle, perhaps due to technical difficulties.

As there is apparently no documentation of neuronal stimulation of the isolated ciliary muscle tissue, the present experiments were carried out in an attempt to investigate the physiological properties of the muscle. This *in vitro* study elucidated the physiological and pharmacological characteristics of ciliary muscle more extensively than did the *in vivo* findings obtained via accommodative response (Helmholtz, 1962; Davson, 1980).

Contraction of smooth muscle is produced either by a direct effect of the electrical current on the smooth muscle, i.e., via depolarization of muscle membrane, or by an indirect effect of autonomic nerve fibres, i.e., by a consequent release of transmitters. The present experiments suggest that the contractile activities of the ciliary muscle are solely dependent on cholinergic nerves and not on depolarization of the muscle *per se*.

Methods

Bovine eyes obtained from a slaughter house were enucleated within 2–3 min of death and immediately

placed in an oxygenated modified Krebs solution and used for study within 30 min. Ciliary muscle strips (5 mm wide and 6 mm long) 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 (Suzuki & Kobayashi, 1982). Sutures were tied to both ends of the muscle strip. A short ciliary nerve approximately 8 mm long was then carefully exposed by stripping off from the sclera and was carefully placed on a pair of coated Ag-AgCl electrodes. The data were obtained from 300 strips.

To investigate the mechanical properties, the tissue was mounted in a 0.5 ml organ bath through which Krebs solution at 36°C flowed continuously, and which was aerated with 95% O_2 and 5% CO_2 (1.5 ml/min). One end of the tissue was tied to a force displacement transducer (FD pickup, model TB612T, Nihon Kohden Ltd.) with a load of about 150 mg. 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 stimulator and a 2 KΩ series resistance (model MSE-3R, Nihon Kohden Ltd.).

Krebs solution was of the following composition (mM):Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134.0, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.5. High K solution (158.8 mM, hereafter termed high K) was prepared by replacing NaCl and $NaHCO_3$ with equimolar amounts of KCl and $KHCO_3$, respectively. The following drugs were used: carbachol, (±)-noradrenaline, (−)-isoprenaline hydrochloride, tetraethylammonium chloride (TEA), physostigmine and neostigmine bromide. All were from Sigma. Atropine sulphate, tetrodotoxin, (±)-propranolol hydrochloride (Sigma) and phentolamine (Regitine, CIBA) were used in order to distinguish the effects of the autonomic nerves from those of the smooth muscle cells. The concentrat of agents used is expressed in g/ml and indicates final concentration, achieved in about 20 s after changing the solutions.

Results

One hour after the incubation of these strips, the preparation usually relaxed and reached a stable tone with a final length of 5–6 mm. Spontaneous contractions were absent throughout.

Carbachol (over 50 ng/ml) generated a sustained contraction, while no contraction was evoked by phenylephrine (0.1 to 5 μg/ml), or by noradrenaline (0.1 to 1 μg/ml), under conditions of pretreatment with 1 μg/ml propranolol. Isoprenaline (0.1–1.0 μg/ml) or noradrenaline (0.1–1.0 μg/ml), produced no relaxation. When the muscle was made to contract with carbachol, isoprenaline produced no relaxation. Thus the bovine ciliary muscle apparently possesses no functional adrenoceptors. Adrenoceptors have been found in the ciliary muscle of several other species (van Alphen, Kern & Robinette, 1965; van Alphen, 1976).

Mechanical responses of the ciliary muscle evoked by electrical stimulation (Figures 1–4)

The short ciliary nerve connected to a ciliary muscle was placed on paired electrodes in the air and stimulated. In some experiments, transmural field stimulation was carried out, where the maximal contraction was usually larger than that obtained by stimulation of the isolated nerve.

A single shock delivered to the nerve provoked a twitch-like contraction. The minimal duration required to evoke a sizable contraction was usually 0.2 ms. The amplitude of contraction became progressively larger with prolongation of the stimulus duration and a maximal contraction could be elicited at 2 ms duration. No further increase in the contractile response was produced by prolongation of stimulus duration up to 500 ms. When the muscle strip was treated with 0.1 μg/ml tetrodotoxin, no contraction was evoked, even when the stimulus intensity was increased (b).

Repeated stimuli produced a summation of contractions (Figure 2 a,b,c). As the frequency

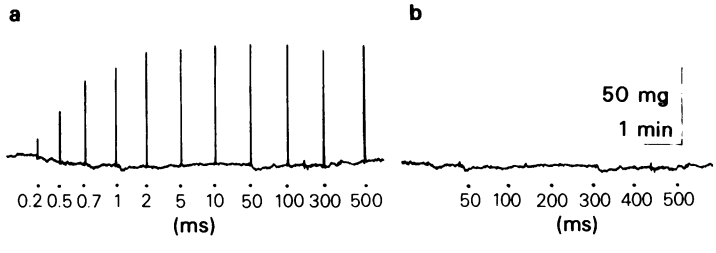

Figure 1 (a) Effects of single electrical stimuli to a short ciliary nerve. (b) Effect of tetrodotoxin on the evoked contractions. Stimulation was applied at the dots. The duration of electrical pulses was increased from left to right in the record. Numbers indicate the duration of single pulses (ms). Note that the peak tension was attained with stimuli of several ms duration.

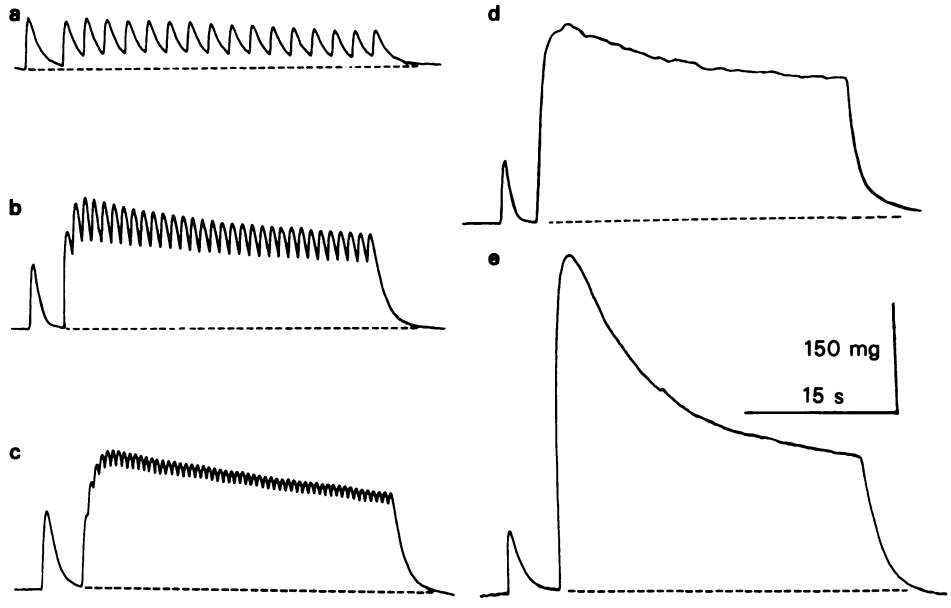

Figure 2 Effects of increasing the frequency of train pulses with constant duration at 0.5 ms on the contractile response. Stimulus frequency was 0.2, 1.0, 2.0, 4.0 and 20 Hz respectively, for the records (a), (b), (c), (d) and (e). The initial contraction in each record shows a 'twitch' due to a single stimulus.

was increased, incomplete tetanus proceeded to a complete tetanus with 4 Hz stimulation (Figure 2d). With a higher rate of repetitive stimuli (e, 20 Hz), the contraction exhibiting a steeper onset, a higher amplitude was elicited, and the muscle strip gradually relaxed, despite continuation of the electrical stimulation. The relaxation was affected neither by the application of propranolol (1 μg/ml), nor by phentolamine (1 μg/ml).

The effects of physostigmine on the mechanical responses to field stimulation are shown in Figure 3. The amplitude of contraction evoked by a single electrical shock (0.5 ms) was increased and the duration of contraction was prolonged when 10 ng/ml physostigmine was added to the Krebs solution (a). The basal tone was elevated independently of electrical stimulation by the application of 0.5 μg/ml physostigmine and the evoked contraction became correspondingly smaller (b). Physostigmine was about ten times as potent as neostigmine. Addition of atropine (0.5 μg/ml) or tetrodotoxin (0.5 μg/ml) completely suppressed the mechanical response evoked by field stimulation, as shown in (c). Elevation of the basal tone in the presence of physostigmine was abolished by pretreatment with atropine. Propranolol (0.1–5.0 μg/ml) or phentolamine (0.1–5.0 μg/ml) produced no change in the amplitude of the evoked contractions to field stimulation.

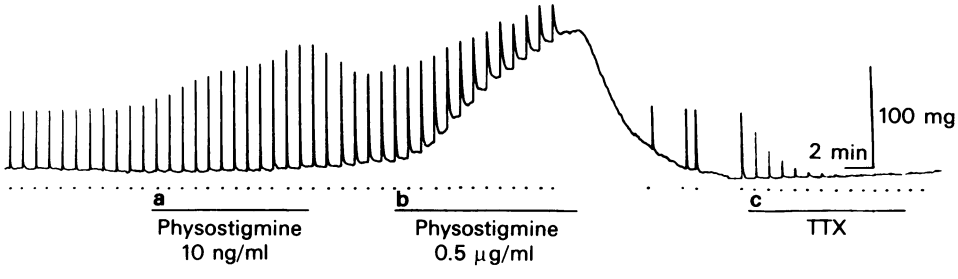

Figure 3 Effects of physostigmine on the electrically-induced contraction of bovine ciliary muscle (single field shock, 0.5 ms, 1 min interval). (a) Potentiation of evoked contractile responses in 10 ng/ml physostigmine. (b) Elevation of basal tone and a prolongation of contraction due to electrical stimulation in 0.5 μg/ml physostigmine. (c) Complete abolition of evoked contractions by 0.5 μg/ml tetrodotoxin (TTX).

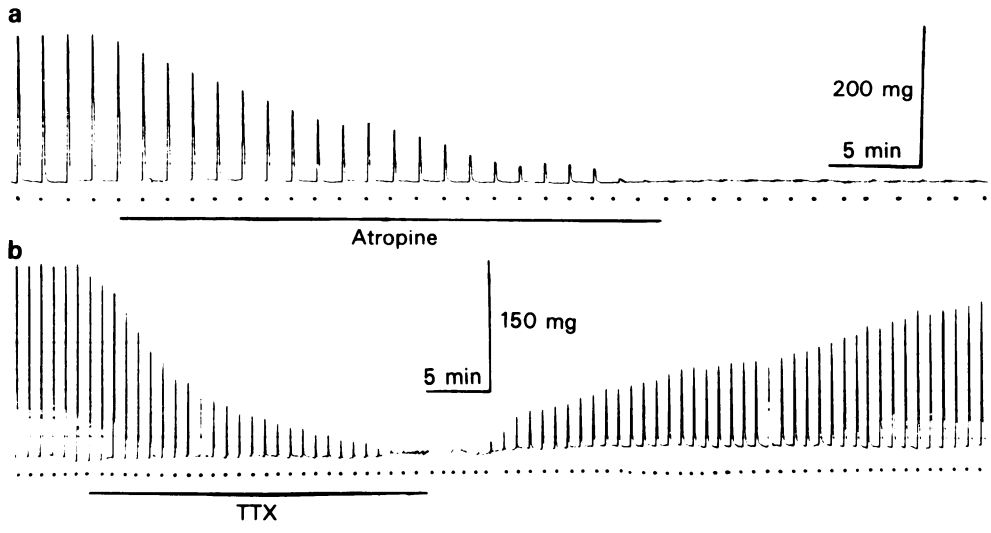

Figure 4 Effects of atropine and tetrodotoxin on the mechanical responses recorded from cilialy muscle. Stimulus conditions (marked by the dots) were fixed at 30 ms, 20 Hz and 60 pulses. (a) Abolition by 0.5 μg/ml atropine. Partial recovery of evoked contraction appeared 3 h after a wash with normal Krebs (not shown). (b) Complete inhibition by tetrodotoxin (TTX) 0.1 μg/ml and fast recovery after washing. Field stimulation was applied.

These results indicate that the bovine ciliary muscle is innervated predominantly by excitatory cholinergic nerves.

Effects of direct stimulation of the ciliary muscle were then investigated using long electrical pulses (20 ms–100 ms) with variable frequencies. Contrary to our expectation, tetrodotoxin or atropine completely suppressed the development of the evoked tension (Figure 4). Mechanical responses to electrical stimulation were not evident, even when the gain of the mechanorecorder was increased to observe the phenomena in greater detail. Tetrodotoxin blocks the transmission of the nerve impulse selectively but does not normally suppress smooth muscle activity (Kuriyama, Osa & Toida, 1967). Ciliary contractions evoked by electrical stimulation are therefore most likely due to excitation of the nerve, particularly the muscarinic receptors, and not by the excitation of muscle cell *per se*, even with long durations which are generally considered sufficient to evoke muscle excitation.

Effects of external K concentration (Figures 5,6)

A contractile response was initiated in the bovine ciliary muscle, when the external K concentration ($[K]_o$) was elevated. As shown in Figure 5, the contracture evoked by 158.8 mM K (isotonic K solution) was composed of an initial phasic component and a following tonic one (a). The amplitude of the phasic contraction was all but equal to the electrically-induced contraction by repetitive pulses (1 ms dura-

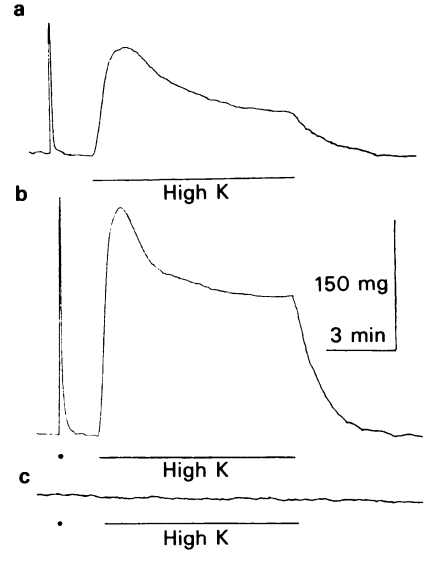

Figure 5 Effects of high K on bovine ciliary muscle in comparison to the preceding response due to electrical nerve stimulation (1 ms, 20 Hz, 60 pulses). (a) A phasic component gradually followed a tonic one. (b) Elevation of K contracture in the presence of 50 ng/ml physostigmine. (c) Contractions were not generated in the presence of 1 μg/ml atropine, either in response to nerve stimulation or in the presence of high K solution.

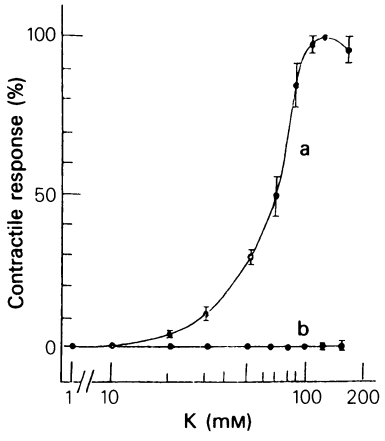

Figure 6 (a) Effects of various concentrations of $[K]_o$ on the mechanical response (ordinate scale). Responses are expressed as percentages of the maximal response to 120 mM $[K]_o$. (b) Inhibition of (a) by the application of 0.5 μg/ml atropine. Concentrations of $[K]_o$ are expressed on a logarithmic scale on the abscissa scale. Vertical lines indicate the s.d. ($n = 12$).

tion, 20 Hz), which yielded a maximal response to nerve stimulation. When 50 ng/ml physostigmine was applied, the amplitude of both the phasic and tonic contractions was increased (b). Administration of 1 μg/ml atropine remarkably depressed the generation of K-contracture (c). The K-contracture, however, exhibited no significant change, in the presence of tetrodotoxin (1–50 μg/ml).

The amplitude of the phasic contraction was plotted against the external K concentration in Figure 6 in order to measure the mechanical threshold of the ciliary muscle. The mechanical threshold in terms of K concentration was about 20 mM, and the maximal contraction was reached with 120 mM $[K]_o$. When atropine was applied, the threshold concentration of $[K]_o$ shifted to 100–110 mM and the maximal contraction was reduced to 1–2% of the control response. This indicates that depolarization of the cholinergic nerve terminals with consequent release of acetylcholine plays a most significant role in the development of K contracture in the ciliary muscle and a negligible role on the muscle *per se*. Propranolol (1–5 μg/ml) or 1 μg/ml phentolamine did not influence the amplitude of the contracture induced by any concentration of $[K]_o$. These contractile effects were reproduced when the experiments were repeated at intervals longer than 30 min. Complete recovery of the tissue was assessed by the contractile amplitude due to electrical stimulation. These results suggest a minor dependency of not only the sympathetic nervous system but also the generation of contraction on the membrane potential.

Effects of tetraethylammonium (Figure 7)

Contractions of smooth muscles are usually poten-

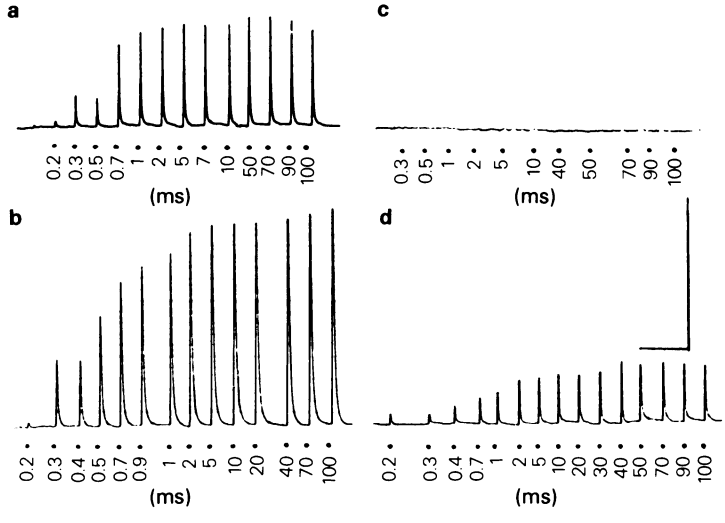

Figure 7 Effects of 20 mM tetraethylammonium (TEA) and atropine on the evoked contractions by field stimulation. Stimulus duration was increased from left to right in the record. (a) Responses in Krebs solution to a single electrical stimulation. (b) Responses to the same stimulation as in (a) in the presence of 20 mM TEA. Potentiation occurred regardless of the pulse duration. (c) Complete inhibition by 1 μg/ml atropine (cf. (a)). (d) Effects of 20 mM TEA added in the presence of 50 μg/ml atropine (cf. (c)). Reduced but detectable contractions were generated.

tiated with application of TEA (see Bolton, 1979). TEA (1 to 30 mM) did not influence the baseline tonus and spontaneous mechanical activity was not generated throughout. The substitution of TEA by 60 mM sucrose in the normal Krebs was without effect. Figure 7 shows twitch responses evoked by various durations of electrical field stimulation (0.2–100 ms) before (a,c) and after application of 20 mM TEA (b,d). The amplitude of contraction was enhanced by the application of 5–20 mM TEA (b vs. a), and the amplitude was maintained to some degree when 1–50 μg/ml atropine was applied in addition (d vs. c). Tetrodotoxin (0.1–10 μg/ml) had the same effect as atropine. This tendency was essentially the same, whenever the muscle strip was stimulated singly or repetitively. Therefore, it seems probable that TEA acts pre-junctionally as well as post-junctionally, in this tissue. Electrical pulses shorter than 0.3 ms remained incapable of generating contractions in the presence of atropine, yet the contraction with a fairly large amplitude was generated in a graded manner as the duration was increased from 0.3 to 2 ms and then saturated. The stimulation frequencies required for complete tetanus ranged from 3–4 Hz.

Discussion

The present experiments have shown that bovine ciliary muscle contractions induced by high K or evoked by electrical stimulation, even with longer pulses, were greatly potentiated in the presence of physostigmine or neostigmine and were completely inhibited by the application of atropine. Thus, depolarization alone did not generate a contraction of this muscle. These findings suggest that these contractions do not depend on depolarization in muscle cells but are rather controlled by acetylcholine release from the short ciliary nerve terminals. The ciliary muscle seems to be characterized by the lack of excitability of muscle cell membrane *per se*, with any electrical long pulses or depolarization caused by high K.

Nevertheless, high K produced a contraction in the presence of tetrodotoxin, although atropine suppressed the K contracture almost completely. Tetrodotoxin suppresses nerve activity without affecting the smooth muscle (Kuriyama *et al.*, 1967), however, it does not block the transmitter release via depolarization of the nerve terminals, in the presence of external calcium (endplate, Katz & Miledi, 1966). If this notion is applied to autonomic nerve endings, it supports the idea that K contracture of the ciliary muscle depends on neuronal elements. It should be noted that the contribution of neuronal involvement was considerably greater in the ciliary muscle than in other tissues. Why the ciliary muscle cell does not contract when depolarized remains to be determined.

TEA reportedly suppresses K conductance (Armstrong, 1974; Bolton, 1979; Bülbring *et al.*, 1982) and induces an action on smooth muscles that do not normally generate action potentials (sheep carotid artery: Keatinge, 1975; rabbit ear artery: Droogmans, Raeymaekers & Casteels, 1977; trachea: Kirkpatrick, 1975; Suzuki & Kuriyama, 1976). In the present experiment, TEA markedly enhanced the electrically-induced ciliary muscle contraction which was partially inhibited by atropine. The effects of TEA could not be completely overcome by increased concentrations of atropine (50 μg/ml) or tetrodotoxin (10 μg/ml). Thus, TEA potentiates acetylcholine release through a prolongation of the presynaptic spike, on the one hand (Lundh, Leander & Thesleff, 1977). On the other hand, atropine-resistant or tetrodotoxin-insensitive ciliary contraction in the presence of TEA indicates that TEA acts on the smooth muscle cell, either by direct muscle stimulation evoking action potentials, or by displacing tightly bound calcium, thereby modifying sodium conductance and directly, increasing excitability (Beaulieu, Frank & Inoue, 1967). The direct depolarizing action of TEA may be insufficient to cause spiking unless electrical stimulation is also applied, since the ciliary muscle membrane may exhibit a strong rectification.

In general the tension development in smooth muscles can be generated by (1) action potentials (e.g. taenia coli: Bülbring, 1954), by (2) excitatory junction potentials (e.g. vas deferens: Holman, 1970) or by (3) slow potential changes (e.g. stomach fundus: Osa & Kuriyama, 1970; sling muscle: Beck & Osa, 1971). Furthermore, contractions can also be generated by transmitter actions independent of change in the potential, in some muscles (e.g. carotid artery: Mekata, 1971; aorta: Mekata, 1974; trachea: Kirkpatrick, 1975; Suzuki & Kuriyama, 1976).

The quiescent ciliary muscle belongs to the last group (carotid artery, aorta and trachea type). Taking into account the mechanical activities from incomplete to complete fusion, according to the stimulus frequency, the ciliary contraction may be mediated via excitatory junction potentials, yet these potentials alone would be ineffective in generating the contraction. Spontaneous release of transmitter may also be present, since higher doses of physostigmine increased the basal tone, possibly indicating the release of acetylcholine from unstimulated nerve terminals. It cannot be ruled out that the response to exogenous acetylcholine is accompanied by a calcium release in the muscle cells known as pharmacomechanical coupling (Somlyo & Somlyo, 1968). The ciliary muscle does not appear to respond by generating the spike, since the K contracture was abolished in the presence of atropine. Since accommodation is a fine and rapid movement, it appears odd that this muscle does not respond via action potentials.

Although the muscle is innervated by autonomic nerves (Ruskell *et al.*, 1979; Davson, 1980) and has muscarinic receptors, the bovine ciliary muscle also seems to behave like a twitch skeletal muscle fibre. If it is a 'twitch' muscle with virtually no tonic component, high-K may depolarize too slowly to generate action potentials and tension, as is the case in skeletal muscle fibres (e.g. Bolton & Jones, 1977). Furthermore, the direction of the potential field across the muscle fibres may be crucial and not optimum in the experimental preparation used, due to the multi-unit nature of the muscle. If so, the absence of K-contracture and evoked contractions due to longer pulses (20–100 ms) may not be regarded as conclusive evidence against action potential generation.

Ciliary muscle is a technically difficult tissue to study and we were not able to record the potential change in response to electrical stimulation. The sucrose-gap technique also could not be used to assess electrical activities, due to the multi-unit nature of the muscle cells. Electrical features are thus open to question and further studies are required to clarify the depolarization-contraction coupling mechanism of the ciliary muscle. Species differences have to be studied, because similar results were obtained in monkeys (unpublished data). As calcium plays a central role in excitation-contraction coupling (see Bolton, 1979), experiments of this nature should be useful for clarification of the mechanics of intraocular smooth muscles.

I wish to thank Profs. T. Osa and S. Kobayashi (Departments of Physiology and Ophthalmology, respectively, Yamaguchi University School of Medicine) for critical discussions, Dr T. Kuwabara of N.I.H. for comments and M. Ohara of Kyushu University for preparing the manuscript.

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(Received September 13, 1982.
Revised October 19, 1982.)