Cholinergic Inhibitory Response in the Bovine Iris Dilator Muscle

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Isometric tension changes of the bovine iris dilator muscle were investigated in vitro. Electrical field stimulation (0.03–1 msec duration) produced a relaxation, which was abolished by the addition of tetrodotoxin, thereby suggesting a neuronal origin. Marked relaxation was also initiated in high K solution. Both of these relaxations were potentiated by neostigmine, while atropine abolished the relaxations. Exogenous application of acetylcholine or carbachol produced dose-dependent relaxations that were not antagonized by adrenoceptor blocking agents. These observations indicate that the relaxations induced either by electrical nerve stimulation or by high K are mainly mediated via excitation of cholinergic nerves and that the consequent release of acetylcholine relaxes the muscle. The cholinergic inhibitory mechanism may be accompanied by hyperpolarization of the muscle membrane or be due to a direct transmitter action via "pharmacomechanical coupling." Adrenergic agonists and high K produced weak contractions even when the muscle relaxed with carbachol application. Adrenergic agonists and high K produced weak contractions even when the muscle relaxed with carbachol application. The adrenergic system and the depolarization of the muscle seem to play some role on the motor function of the bovine dilator.

The present experiment suggests that the cholinergic system plays an unexpectedly dominant part in dilation of the bovine pupil, since cholinergic agents produced considerable responses of the dilator muscle, as compared to findings in the case of adrenergic agonists. The inhibitory cholinergic nerves innervating the bovine dilator are closely related to the miotic action of the sphincter muscle. The unexpected inhibitory action of acetylcholine may possibly be a general occurrence among mammals.

Materials and Methods

Bovine eyes obtained from a slaughterhouse were placed in an oxygenated Krebs solution and were usually used for study within 30 min of enucleation. Under a dissecting microscope, the cornea was cut away and isolated dilator preparations were obtained by radial cutting of the superior and inferior sides of the iris (3 mm wide and 4 mm long). The lateral sides of the iris were used occasionally. Sutures were tied to both ends of the muscle strip. Mechanical recordings (recticorder, model RGC-4028, Nihon Kohden, Ltd.) were made with an isometric tension transducer (FD pickup, model TB612T, Nihon Kohden, Ltd.) with a load of 30 mg. The muscle strip was stimulated electrically (stimulator, model SEN-3201, Nihon Kohden, Ltd.) with supramaximal pulses (50 V) by

The iris contains two types of muscles, the sphincter pupillae and the dilator pupillae. These are two of the very few muscles derived from neural ectoderm.1 Evidence that the mammalian iris sphincter is controlled in part by inhibitory adrenergic nerves, as well as dominant excitatory cholinergic nerves, has been clarified by histologic,2,3 pharmacologic,4-6 and physiologic investigations.7,8 However, attention has not been given to the neural effects on the motor function of the isolated dilator muscle, although some investigators suggested the possible cholinergic participation in the dilator muscle, eg, in response to exogenous acetylcholine weak contraction occurs in the rabbit dilator,6 and in the cat dilator, weak relaxation is followed by contraction.9,10 Thus, it is generally considered that the iris dilator is excessively innervated by excitatory adrenergic nerves.

The purpose of our present work is to investigate the physiologic properties of the dilator muscle, particularly in relation to the effects of nerve stimulation and autonomic agents. The effects of external potassium (K) concentration were also investigated. The action and mode of the unexpectedly extensive involvement of inhibitory cholinergic system and contribution of the excitatory adrenergic system in the bovine dilator are presented. The inhibitory mechanism due to acetylcholine is also discussed.

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a pair of Ag-AgCl electrodes set up on each side of the preparation. Experiments were performed in a 0.1-ml organ bath, through which 37° C Krebs solution flowed continuously at a rate of 0.6 ml/min with micro-tube pump (Tokyo Rikakikai Co., Ltd.), and it was aerated with 95% O₂ and 5% CO₂.

The Krebs solution was of the following composition (mM): Na⁺, 137.4; K⁺, 5.9; Mg²⁺, 1.2; Ca²⁺, 2.5; Cl⁻, 134.0; H₂PO₄⁻, 1.2; HCO₃⁻, 15.5 and glucose, 11.5. High K solution (158.8 mM) was prepared by replacing NaCl and NaHCO₃ with equimolar amounts of KCl and KHCO₃, respectively. The following drugs were used: acetylcholine- and carbachol-chloride, DL-norepinephrine, phenylephrine-hydrochloride, L-isoproterenol-hydrochloride, eserine, neostigmine bromide, tetrodotoxin,11 atropine-sulfate and DL-propranolol-hydrochloride. All were from Sigma Chemical Co. (St. Louis, MO). Phentolamine (Regitine, CIBA) was also used. The final concentration of agents is expressed in mole/l(M). Other details were as described previously.8,12

Results

Thirty minutes after incubation of the bovine dilator strips, the preparation usually relaxed and thereafter slightly contracted to a stable intrinsic tone. Spontaneous mechanical activity was absent throughout.

Effect of Autonomic Drugs (Fig. 1)

Exogenous application of norepinephrine unexpectedly elicited only a weak contraction of the bovine dilator muscle (Fig. 1a). Contraction generated either by 10⁻⁵ M norepinephrine or 10⁻⁶ M phenylephrine was antagonized by 10⁻⁵ M phentolamine. The response did not convert into any detectable relaxation of the muscle, despite the evidence that 5 × 10⁻⁷ M isoproterenol itself generated a weak relaxation. Neither phentolamine (5 × 10⁻⁶ M) nor propranolol (10⁻⁶ M) had any effect on the resting tension.

Figure 1b shows typical responses and the dose-relaxation curve for carbachol. Application of carbachol produced a prompt relaxation of the dilator muscle in concentrations over 5 × 10⁻⁹ M. The relaxation was well maintained for several minutes until wash out with normal Krebs solution. The amplitude of relaxation induced by carbachol was much greater than by norepinephrine. Relaxation reached a maximum at a concentration of 5 × 10⁻⁶ M. Relaxation was also initiated with application of 5 × 10⁻⁸ - 10⁻³ M acetylcholine. Reproducible responses to acetylcholine could be obtained repeatedly for 4 hrs. The acetylcholine-induced relaxation was enhanced by 10⁻⁷ M neostigmine and antagonized by 10⁻⁶ M atropine.

Effect of Electrical Field Stimulation (Figs. 2-5)

The field stimulation produced relaxation (Fig. 2). Minimal duration required to evoke a sizable relaxation with a single stimulation was 0.05 msec and the

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Fig. 1. Dose-response curve of the bovine dilator for norepinephrine (a, open circles) and carbachol (b, closed circles). Responses are expressed as a percent of the response of the amplitude of 5 × 10⁻⁶ M carbachol-induced relaxation (ordinate). Plus and minus signs indicate contraction and relaxation, respectively. Specimens used were 11 muscle strips for norepinephrine, and 13 for carbachol. Vertical bars are twice the standard deviation. Inset: Contraction and relaxation of the dilator induced by cumulative application of norepinephrine and carbachol are originally shown in (A) and (B), respectively. Vertical bar: 30 mg, horizontal bar: 3 min.

Fig. 2. Relaxation induced by single electrical pulses with variable durations. Relaxation is expressed as a percent of the amplitude of the relaxation evoked by electrical stimulation with 3-10 msec pulse duration (n = 9). Inset: Typical responses induced by electrical stimulation (a; single pulses; b; 30 Hz, 60 pulses).
elicited relaxation reached a maximum at 2 msec duration (a). Repeated stimulation elicited a similar but a somewhat enhanced relaxation (b). Relaxation was summated at more than 1 Hz, and the response all but reached a maximum at 20–60 Hz (b). During the course of electrical stimulation, the so-induced relaxation was well sustained. At frequencies over 20 Hz, the evoked relaxation was preceded occasionally by a small contraction, and this contraction tended to be inhibited by prior application of $10^{-6}$ M phenotolamine.

The effects of autonomic blocking agents on the relaxation to field stimulation were then studied (Fig. 3). Evoked relaxations were not antagonized by propranolol (a), yet the addition of atropine completely suppressed the electrically induced relaxation (c).

Figure 4 deals with the effects of neostigmine on the mechanical responses to electrical stimulation. When $3 \times 10^{-8}$-$3 \times 10^{-7}$ M neostigmine was added to the Krebs solution, the amplitude and the duration of the evoked relaxation were increased and prolonged, respectively (a, b). In concentrations over $10^{-6}$ M, the basal tone decreased dose dependently (c, d) and the evoked relaxations became inversely smaller (c). With application of $3 \times 10^{-6}$ M neostigmine, the relaxations were finally abolished (d). Eserine was about five times as potent as neostigmine. Decrease in the basal tone in the presence of neostigmine was abolished by pretreatment with atropine. Effects of neostigmine and atropine on the evoked responses to various stimulus frequencies are shown graphically in Figure 5.

**Effect of external K concentration (Fig. 6)**

Raising the external potassium concentration from 5.9 to 158.8 mM initiated relaxation, and the decline was gradual (Fig. 6a). The high K-induced relaxation was little influenced either by $10^{-6}$-$10^{-5}$ M propranolol or by $10^{-6}$ M phenotolamine. However, addition of atropine suppressed the K-induced relaxation (Fig. 6b), and the amplitude of the K-induced relaxation was potentiated by $5 \times 10^{-8}$ M neostigmine.

Figure 7 shows the effects of norepinephrine and atropine on the relaxation to field stimulation.
high K under conditions of pretreatment with carbachol. The muscle that relaxed with application of carbachol (5 × 10⁻⁹–10⁻⁷ M) and 5 × 10⁻⁷ M tetrodotoxin did not contract in the presence of norepinephrine (5 × 10⁻⁸–10⁻⁶ M) and a dose of over 5 × 10⁻⁶ M norepinephrine was required to induce contraction (Fig. 7a). The amplitude of contraction caused by norepinephrine (5 × 10⁻⁶–10⁻⁵ M) was smaller than that of the 5 × 10⁻⁶ M carbachol-induced relaxation. In such a fully relaxed muscle, high K produced a weak contracture (Fig. 7b).

The bovine iris has an oval-shaped pupil, and the horizontal axis is longer than the vertical one (Fig. 8 left). The bovine sphincter muscle reacted in the same way, independently of the sphincter area cut away from a preparation. However, in the bovine dilator, the superior and inferior strips were far more sensitive both to drugs and to electrical stimulation (a), than were tissues from the lateral sides (c). The initial contraction in response to electrical stimulation and the marked relaxation to isoproterenol were specific for the preparations (b). The relaxed muscle strip showed a similar tendency. For this reason, the former strips (a) were most often used.

Discussion

The bovine dilator muscle strips usually relaxed with electrical stimulation and occasionally a small contraction preceded the relaxation. These evoked relaxations were suppressed by application of tetrodotoxin, thus suggesting a neuronal origin. These relaxations were potentiated by neostigmine and completely suppressed by atropine, yet they were not antagonized by adrenoceptor blocking agents. Moreover, exogenous acetylcholine or carbachol relaxed the muscle. The cholinergic relaxation evident in this tissue is not considered to be a contamination with the sphincter and/or the ciliary muscle, since cholinergic inhibition has never been observed in these tissues. Therefore, the bovine dilator seems to be mainly controlled by the cholinergic inhibitory system, although a presynaptic effect on release of a non-adrenergic activator cannot be ruled out. The cholinergic relaxation of the dilator may assist in the effect of cholinergic miosis of the sphincter. Acetylcholine can be released spontaneously from nerve terminals or dilator muscle cells per se, without electrical nerve stimulation, as suggested by elevation of the basal tone by atropine and the lowering of the tone by neostigmine.

It was very recently shown that lower doses of acetylcholine relaxes the rat dilator, although this same tissue contracted with application of large doses of acetylcholine (10⁻³ M). In the present study, all concentrations of acetylcholine applied produced only relaxation in the bovine dilator (5 × 10⁻⁸–10⁻³ M). The cholinergic inhibitory mechanism in cattle, therefore, probably plays a far greater role than hitherto assumed. Histologic studies revealed cholinergic nerve terminals in monkey, cat, rabbit, and guinea pig, in addition to the well described adrenergic innervation.

The preparation contracted fully during equilibration in the tissue bath, and further contractile responses to norepinephrine could not be elicited. However, PGF₂α does cause an ample contraction of the same tissue, indicating a preservation of the contractile capacity. Furthermore, a 50 times higher concentration of norepinephrine was required to contract the muscle that relaxed with prior application of cholinergic agents, even then the contraction was much smaller in amplitude than was the relaxation. Thus, although the bovine dilator does spontaneously contract during the incubation, the muscle appears to be far more sensitive to cholinergic than to adrenergic agonists. We cannot definitely rule out the impor-
tance of adrenergic involvement, since the actions of adrenergic agonists, either exogenously administered or endogenously released, may be susceptible to degradation and removal of these agents via metabolism, neuronal or extraneuronal uptake, and to binding to pigment.16,17

It is widely accepted that increasing the external K concentrations gradually depolarizes muscle cell membrane, thereby generating K-contracture. The fully relaxed bovine dilator generated weak contracture. However, without the pretreatment, the response to high K solution was one of relaxation. Furthermore, the K-induced relaxation was all but completely inhibited by atropine. Thus, development of the K-induced relaxation can be explained as a total of a weak contracture of the muscle and a remarkable relaxation due to the released acetylcholine by a K-induced depolarization of the nerve terminals.

In smooth muscle, the inhibitory action of acetylcholine has been noted only in certain vascular smooth muscles (coronary artery18). In cardiac muscle, acetylcholine inhibits the slow inward calcium current and thereby decreases the contractile activity,19 although the baseline tone is not lowered. Recording of the membrane potential change with microelectrodes was technically difficult in the bovine dilator, particularly during the nerve stimulation. The sucrose-gap method also was not feasible, due to the multi-unit nature of these muscle cells. The inhibitory mechanism may be accompanied by hyperpolarization or be due to a direct transmitter action via some "pharmacomechanical coupling" process20 without potential change. It is seriously questioned that the bovine dilator contracts during the physiologic "resting" state, and the active response is composed of nerve-mediated relaxation. The manner in which acetylcholine relaxes the muscle and how the muscle tone is spontaneously elevated in terms of membrane potential and calcium movement are open to question.

It is generally considered that the dilator in mammals is innervated by the excitatory adrenergic system.4-6,9,10,14 Nevertheless, the possibility exists that the cholinergic inhibitory mechanism supports both the excitatory cholinergic miosis of the sphincter by its activation, and possibly the excitatory adrenergic mydriasis of the dilator by its passivity. The inhibitory cholinergic system probably contributes to the control of the adrenergic dilator muscle in other species, though less than in cattle.

**Key words:** iris dilator muscle, cholinergic inhibition, electrical stimulation, adrenergic receptor, cattle

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**References**

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