Br. J. Pharmac. (1985), **85**, 905–911

Pharmaco-mechanical coupling in the response to acetylcholine and substance P in the smooth muscle of the rat iris sphincter

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1 In the rat iris sphincter muscle contractile responses to transmural stimulation consisted of two components, a fast cholinergic followed by a slow non-adrenergic, non-cholinergic (NANC) one. The magnitude of the latter varied widely and was on average 5% of that of the cholinergic component.

2 Exogenous substance P (1 nM-1 μM) produced a concentration-dependent contraction, the maximum amplitude of which was as large as that produced by acetylcholine (ACh).

3 Capsaicin (10 μM) induced a transient contraction only once in each preparation. After the treatment with capsaicin the NANC component disappeared.

4 Neither nerve nor direct electrical stimulation with short pulses elicited any active change in the membrane potential under physiological conditions, but an action potential was triggered by direct stimulation when the extracellular Ca ion was totally replaced by Ba ion. Under the latter conditions spontaneous spike potentials occurred repetitively.

5 ACh and substance P produced a large contraction without modifying the membrane potential. This was also the case in the presence of 5 mM Ba.

6 These results suggest that substance P-ergic innervation may have a far lesser physiological significance than that which has been described in rabbits and that pure pharmaco-mechanical coupling is characteristic of the responses to acetylcholine, substance P, and nerve stimulation in the rat iris sphincter muscle.

Introduction

It is now well established that the motor nerve innervating the mammalian iris sphincter muscle is the parasympathetic nerve. The sphincter region of the iris is densely innervated by cholinergic nerve fibres (Csillic & Koelle, 1965; Ivens *et al.*, 1973; Huhtala *et al.*, 1976), and also by sensory nerves of trigeminal origin having substance P-like immunoreactivity (rat: Miller *et al.*, 1981; Shimizu, 1982; rabbit: Tervo *et al.*, 1982; Tornqvist *et al.*, 1982). Many lines of evidence support the suggestion that the sensory nerve may play a role in myosis probably by releasing substance P under some conditions (Maurice, 1954; Bill *et al.*, 1979; Ueda *et al.*, 1981; Bito *et al.*, 1982; Zhang *et al.*, 1984).

Despite these pharmacological and histochemical studies on the neuronal control of pupillary diameter, little is known of the electrophysiological properties of the cell membrane, and the events of neuromuscular transmission in the mammalian iris muscle. In preliminary experiments in the rat, we obtained resting membrane potentials but could not record any evoked electrical activity from the sphincter region. The cells were assumed to be electrically quiescent. Spontaneous action potentials associated with contractions were, however, recorded from all cells penetrated when Ca ion in the medium was replaced by equimolar Ba ion. This strongly suggested that the steady negative potential recorded in a physiological environment was derived from the smooth muscle cell itself (Imaizumi *et al.*, 1984), although other kinds of cells exist in the iris sphincter (Bülbring & Hooton, 1954).

In the present studies, the electrical and mechanical responses of the cell to exogenously applied acetylcholine (ACh) and substance P, and neuromuscular transmission during transmural nerve stimulation were investigated in the rat iris sphincter muscle.

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Methods

The iris was isolated from the eye of male Wistar rats (350–550g; Narita & Watanabe, 1981). The isolated ring preparation was mounted horizontally in an organ bath (4 ml) for tension recording. The bath was continuously perfused at a rate of 2 ml min^{-1} with Krebs solution of the following composition (mM); NaCl 112.0, KCl 4.7, $CaCl_2$ 2.2, $NaHCO_3$ 25.0, $MgCl_2$ 1.2, KH_2PO_4 1.2 and glucose 14.0. Phentolamine (1 μM) was normally incorporated in the Krebs solution to inhibit adrenergic effects. The solution in the bath was maintained at 36–37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). Ba-Krebs solution was prepared by replacing all Ca with Ba and 1 μM atropine was incorporated.

To stimulate the muscle (pulse duration, 5–10 ms) and the nerves (pulse duration, 0.1–0.3 ms) transmurally, a pair of platinum bars (500 μm in diameter and 10 mm in length) were used. When only tension was recorded, the stimulating electrodes were placed in parallel on either side along the muscle and the distance between them was approximately 2 mm. The intensity of stimulus current used was maximally 250 mA.

When both electrical and mechanical responses were simultaneously recorded, the muscle was fixed at one end using a piece of V-shaped rubber membrane in the bath (2 ml), as shown in Figure 1. The rat iris sphincter musculature is very thin (ca 50 μm) and is easily torn. The rubber membrane fixed only a small portion of sphincter muscle without causing damage and prevented displacement of the microelectrode, even when muscle contracted. In these experiments, electrical stimuli were applied through a pair of platinum needle electrodes, arranged as shown in Figure 1.

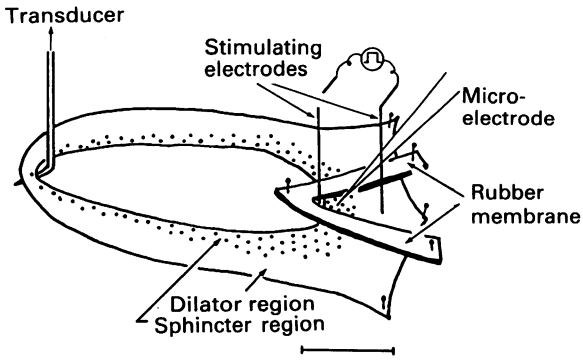

Figure 1 Experimental arrangement for simultaneous recording of electrical and mechanical responses in the rat isolated iris sphincter muscle. The horizontal bar corresponds to about 1 mm by eye measurement.

The contractile response was measured isometrically with a strain gauge transducer (Narita & Watanabe, 1981). The electrical response of the membrane was recorded with glass microelectrodes filled with 3 M KCl, and with a resistance between 30 and 60 MΩ. The microelectrode was inserted into the sphincter muscle cell from the posterior side. All electrical signals were amplified, displayed on a signal-processing oscilloscope (ATAC-350: Nihon-Kohden Ltd, Tokyo) and recorded on pen recorders (B-281 HS: Rika-Denki, or RECTI HORIZ: NEC San-ei Inst. Ltd) or stored on magnetic tape (MR-10; −1.0 dB at 2.5 kHz: TEAC Ltd).

Drugs were applied directly into the bath after cessation of the perfusion. The medium in the bath was continuously stirred by aeration, and the drug reached the preparation at a final concentration within 3 s.

The following drugs were used: acetylcholine chloride and atropine sulphate (Wako Pure Chemicals, Ltd, Tokyo), capsaicin and substance P (Sigma Chemical Co., St Louis), phentolamine mesylate (Ciba-Geigy Japan Ltd), and tetrodotoxin (TTX; a gift from Prof. S. Okuda, The University of Tokyo). Capsaicin (2 mM) was dissolved in the distilled water containing 43% (v/v) alcohol. The final concentration of alcohol in the bath was 0.2% (v/v) or less.

Values are expressed as means ± s.e.mean. The signficance of the difference between values was evaluated using Student's *t* test.

Results

Contractile responses to electrical stimulation

The rat iris sphincter muscle rapidly contracts in response to transmural stimulation (Narita & Watanabe, 1981). Figure 2a shows a typical contractile pattern to repetitive stimulation (0.1 ms in duration at 20 Hz) in the presence of 1 μM phentolamine. The contraction consisted of two components, which will be referred to as fast and slow components. The fast component was large and abolished by an application of 1 μM atropine, while the slow component was atropine-resistant. Additional application of 2 μM TTX completely inhibited the contractile response to nerve stimulation in 9 out of 16 irides (Figure 2a). This indicates that the fast component is cholinergic and the slow may be a non-adrenergic, non-cholinergic (NANC) component. The height of the slow component varied (0–5.3 mg) and in 5 out of the 16 irides the slow component could not be detected. The mean amplitude was only 4.8 ± 1.3% (16 irides) of that of the fast component.

As capsaicin is known to release substance P from sensory nerve ending (Theriault *et al.*, 1979), we

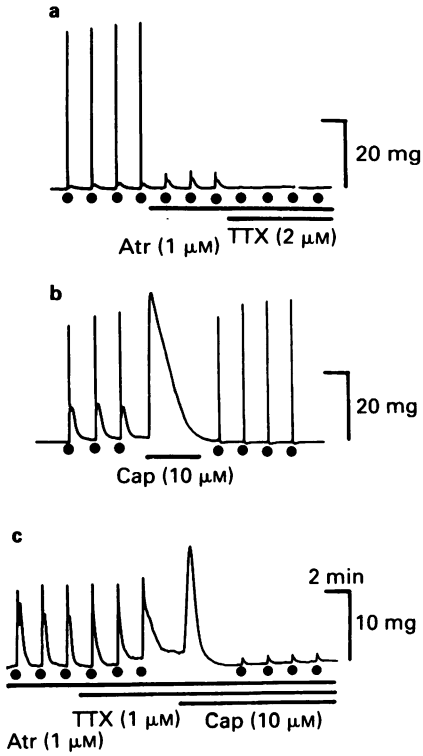

Figure 2 Typical contractile responses induced by electrical stimulation in the rat isolated iris sphincter muscle. (●) Stimuli were applied for 2 s every 2 min. (a) The effects of 1 μM atropine (Atr) and 2 μM tetrodotoxin (TTX), and (b) 10 μM capsaicin (Cap), on the response to electrical stimulation (0.1 ms in duration, 20 Hz in frequency) are shown. (c) Shows the effect of capsaicin (10 μM) on the response which remained in the presence of atropine (1 μM) and TTX (1 μM) during transmural stimulation (0.3 ms in duration, 20 Hz in frequency). The bar under each trace indicates the period of drug application.

studied the effect of capsaicin on the fast and slow components during electrical stimulation. Capsaicin (10 μM) induced a transient rapid contraction (39.0 ± 5.6 mg; 6 irides) which decayed to the resting level after 3.8 ± 0.4 min (6 irides). This response was not affected by 1 μM atropine and 1 μM TTX (42.3 ± 6.4 mg, 4 irides). After the transient response to capsaicin subsided, the slow component disappeared without any change in the fast component (Figure 2b). Even in the preparation where the slow component could not be detected, capsaicin was able to produce the phasic contraction (33.1 ± 5.7 mg).

Figure 2c shows the contractile response to stimulation by slightly longer pulses (0.3 ms) in the presence of 1 μM atropine and phentolamine. Under these conditions only the slow component was present with a

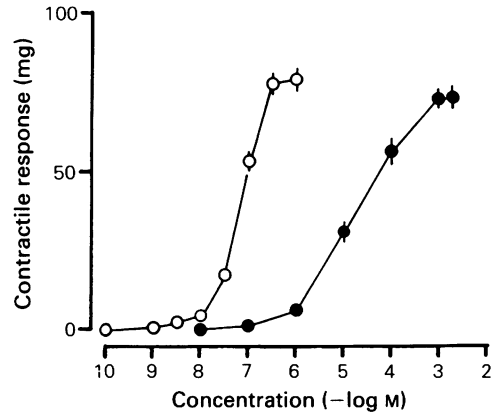

Figure 3 Concentration-response curves for acetylcholine (●) and substance P (○) in the rat iris sphincter muscle. Data shown are the means, and vertical lines represent s.e.mean (when larger than the symbol), of 5 irides.

shoulder on the falling phase. TTX (1 μM) abolished only the shoulder of the contraction. We have already shown that the TTX-insensitive contraction is readily elicited in the rat iris sphincter by electrical stimuli of duration not shorter than 0.3 ms (Narita & Watanabe, 1981). However, in the present study it was found that, after treatment with 10 μM capsaicin, this component was depressed to 18.5 ± 7.1% (6 irides; $P < 0.05$).

Responses to exogenous acetylcholine and substance P

As shown in Figure 3, exogenously applied substance P at concentrations between 1 nM–1 μM produced a

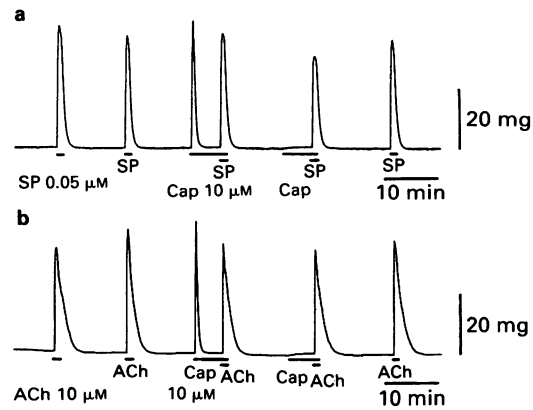

Figure 4 Effects of 10 μM capsaicin (Cap) on the contractile responses induced by (a) 0.05 μM substance P (SP) and (b) 10 μM acetylcholine (ACh). Each drug was applied at 15 min intervals and washed out at the end of the bar under the traces.

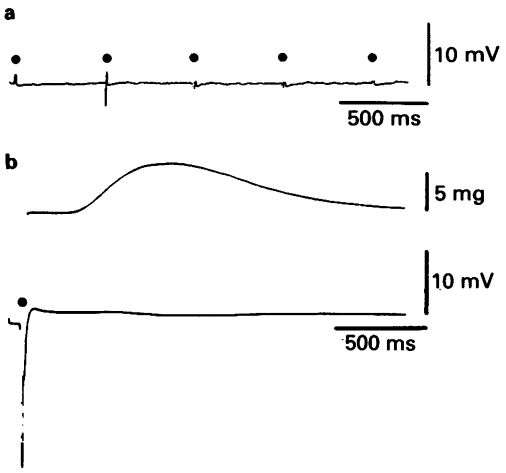

Figure 5 Electrical responses of iris sphincter muscle cells to electrical stimulation. (a) Electrical pulses of 0.3 ms in duration were applied at 500 ms intervals. Stimulus artifacts are seen. Two intracellular recordings of stimulating polarity switched were obtained from the same cell and were averaged. (b) The lower trace shows the response to a single pulse (5 ms in duration) and the upper trace shows simultaneous recording of tension development (mean of eight recordings). Traces (a) and (b) were obtained from two different cells, and membrane potential was − 60 mV and − 57 mV, respectively.

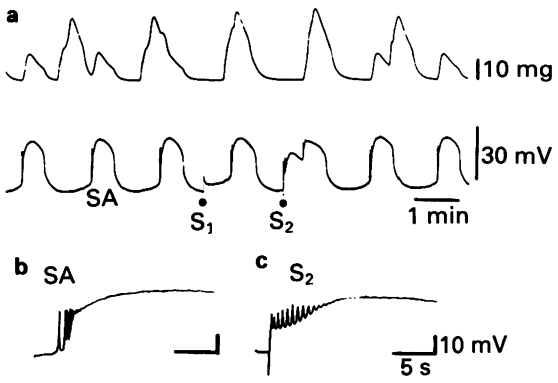

Figure 6 Spontaneously generated spike potentials and electrically triggered spike potential in Ba-Krebs solution. (a) The lower trace shows electrical phenomena and the upper trace shows concomitant contractions. In Ba-Krebs the cell membrane depolarized up to − 46 mV. A single intense pulse (10 ms in duration) was applied at S_1 and S_2. S_2 (15 V), but not S_1 (10 V), was sufficient to produce a spike potential. (b) and (c) Traces at faster recording speed of spontaneous activity (SA) and triggered spike (S_2), respectively.

graded contraction which reached a maximum at 1 μM with a value of 71.4 ± 3.1 mg (5 irides). This was approximately equal to that seen with ACh (67.5 ± 3.1 mg; 5 irides).

Figure 4 shows the effect of capsaicin on the substance P- and ACh-induced contractions. In this muscle, reproducible contractions could be induced by repeated application of a moderate concentration of substance P (Figure 4a) and ACh (Figure 4b), 0.05 μM and 10 μM, respectively. The response to capsaicin was, however, seen only to the first application (Figure 4a,b). The amplitudes of contraction induced by substance P (Figure 4a; 33.2 ± 7.2 mg; 4 irides) and ACh (Figure 4b, 36.4 ± 0.7 mg; 4 irides) in the presence of 10 μM capsaicin were not significantly different from the corresponding responses of 34.1 ± 6.6 mg and 40.9 ± 3.7 mg, respectively, occurring in the absence of capsaicin. These findings strongly suggest that as in other tissues, capsaicin induced a contraction not by a direct action on the iris muscle, but rather by a release, possibly from the sensory nerves, of substance P or related peptides. Moreover, it is very likely that the slow component of the response to transmural electrical stimulation is due to the release of substance P from the same pool that is sensitive to capsaicin treatment since the slow component also disappeared after treatment with capsaicin (10 μM). In addition, it was striking that the TTX-insensitive response was also decreased after capsaicin treatment (Figure 2c). Thus, it may be that substance P is directly released from sensory nerve terminals, by field stimuli, independent of conduction of an impulse along the nerve.

Effects of field stimulation on the membrane potential

The membrane potential in smooth muscle cells of the rat iris sphincter was quite stable at − 57.6 ± 0.5 mV (from 51 cells in 23 irides). No spontaneous electrical activity was found as has been described previously (Imaizumi *et al.*, 1984).

Figure 5 shows a trace of the membrane potential during transmural electrical stimulation. Surprisingly, no change in membrane potential, neither excitatory junction potential nor action potential, was evoked by nerve stimulation of 0.3 ms duration (20 cells, Figure 5a). A slight hyperpolarization of several millivolts in amplitude was occasionally evident, but with a latency of several hundred milliseconds after the stimulus. Since such a pattern of potential fluctuation was not always seen, even in the same cell and the latency was long, it is likely to be an artifact resulting from the contraction. Furthermore, a long pulse (5 ms duration; 18 cells, Figure 5b) also failed to evoke a spike potential, even when a contraction occurred. Thus, the membrane of the rat iris sphincter muscle may not be electrically excitable under physiological conditions. A spike potential was, however, triggered by a long pulse (10 ms duration) in Ba-Krebs solution;

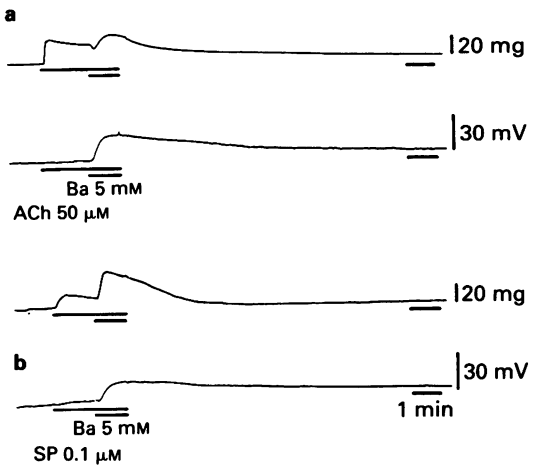

Figure 7 Electrical and mechanical responses of muscle cells to (a) exogenous acetylcholine (ACh; 50 μM), (b) substance P (SP; 0.1 μM) and Ba. Ba 5 mM was successively added to each preparation. Upper and lower traces: mechanical and electrical responses, respectively. The bar under each trace indicates period of drug application. Records (a) and (b) were obtained from different preparations.

spontaneous spike potentials also occurred (Figure 6). The shape of the evoked spike in Ba-Krebs was similar to that of the spontaneously generated spikes (Figure 6b,c). In this preparation, the triggered spike potential was not accompanied by a contraction, probably because the triggered spike did not propagate to the region for tension recording (see Methods).

Effects of exogenous acetylcholine and substance P on the membrane potential

Since our findings suggested that ACh and, probably, substance P released from nerve terminals are responsible for the contraction induced by transmural nerve stimulation in the rat iris sphincter muscle, the effects of bath-application of these two substances on the membrane potential were examined. ACh 50 μM and substance P 0.1 μM, which produce 70–80% of the maximum response did not modify the membrane potential (Figure 7). A subsequent application of 5 mM Ba, however, invariably depolarized the cell membrane and a further contraction was superimposed on the drug-induced contraction. Results of a series of these experiments are summarized in Table 1. Both ACh and substance P had no significant effect on either the membrane potential or the depolarization caused by Ba.

Discussion

We found that in the rat iris sphincter electrical stimulation, ACh and also substance P which both may operate as an excitatory transmitter in this muscle, induced large contractions without depolarizing the cell membrane. That membrane potentials recorded might have been from non-muscle cells (Bülbring & Hooton, 1954) can be excluded by the following observations: (1) in cells where no detectable change in the membrane potential had occurred to either ACh or substance P, Ba invariably elicited membrane depolarization, followed by a contraction. (2) Spontaneous action potentials together with a contraction were recorded from the cells in Ba-Krebs solution, as previously described (Imaizumi *et al.*, 1984). These results strongly suggest that the contractions induced pharmacologically or by nerve stimulation are not mediated by modification of membrane potential.

Tension development without or with little modification of membrane potential has been found in other smooth muscles, for example in large arteries when the chemical transmitter, noradrenaline, is exogenously applied (pulmonary artery: Su *et al.*, 1964; Casteels *et al.*, 1977; ear artery: Droogmans *et al.*, 1977). However, in the ear artery other investigators have argued that membrane depolarization by noradrenaline is apparent and contributes to tension

Table 1 Effects of acetylcholine (50 μM), substance P (0.1 μM) and barium chloride (5 mM) on the resting membrane potential (mV)

Drug	*Drug free*	*Drug*	*Drug + Barium (5 mM)*	n
Acetylcholine (50 μM)	− 58.3 ± 1.7	− 57.0 ± 1.7	− 35.8 ± 1.7	4
Substance P (0.1 μM)	− 58.8 ± 3.0	− 56.0 ± 3.3	− 36.8 ± 0.9	5
Barium (5 mM)	− 58.1 ± 1.1	− 34.7 ± 1.4		8

Each value was obtained only when the microelectrode was maintained within the same cell throughout successive applications of drugs, as shown in Figure 7, or with a single application of Ba. Results show means ± s.e.means of *n* number of observations.

development (Trapani *et al.*, 1981). Thus, the relationship between membrane potential and tension development during the application of an agonist is not completely clear in this artery and is probably the same in other large arteries. A common phenomenon has, however, been observed in these electrically quiescent smooth muscles; it is that electro-mechanical coupling is advanced when GK is decreased in the presence of tetraethylammonium (Haeusler & Thorens, 1980) or Ba ion (Harder & Sperelakis, 1978). In contrast to these observations, in the rat iris sphincter the amount of depolarization produced by Ba was not altered by the addition of ACh or substance P, indicating that these compounds do not effectively modify the membrane potential even under these conditions. Therefore, the response to chemical transmitters observed in the present experiments may be most typical of the pharmaco-mechanical coupling described by Somlyo & Somlyo (1968).

Although no experiment was undertaken to elucidate the reason why ACh and substance P did not modify the membrane potential in the present study, at least two possibilities can be considered. One is that both agents do not alter the ion permeability of the cell membrane to an extent sufficient to modify the membrane potential in this muscle. Another is that they modify membrane permeability to more than one species of ions in such a manner that the net current amounts to zero. The second has been postulated for the mechanism of pharmaco-mechanical coupling in arteries (Casteels *et al.*, 1977; Droogmans *et al.*, 1977). In the present studies, contraction induced by Ba was preceded by membrane depolarization, probably because the ability of Ba to reduce GK (Hermsmeyer & Sperelakis, 1970; Armstrong *et al.*, 1982). Moreover, as mentioned above, ACh and substance P did not effectively modify the membrane potential even when GK was decreased in the presence of Ba, suggesting that the first proposition is to be favoured. More detailed experiments are required to elucidate the mechanism.

We have previously found an anomalous stabilizing action of Ca ion on the cell membrane of the rat iris sphincter muscle (Imaizumi *et al.*, 1984). In the present studies, a spike potential was triggered by an electrical current in Ba-Krebs solution but not in the presence of extracellular Ca. This suggests that extra- and/or intracellular Ca ion is responsible for the electrical inexcitability of the membrane.

In addition to the dominant cholinergic mechanism, a NANC mechanism may be involved in the contractile response of the rat iris sphincter muscle to transmural nerve stimulation. In the rabbit isolated iris, a similar but more significant NANC component has been identified. Moreover, it has been assumed that both NANC- and capsaicin-induced responses can be attributed to substance P released from sensory nerves (Ueda *et al.*, 1981; Zhang *et al.*, 1982, 1984). The results in the present study suggest that such may also be the case for the rat iris sphincter.

However, in our previous work (Narita & Watanabe, 1981), we did not recognize the slow component in response to nerve stimulation. This may have been because the atropine-resistant component in the rat iris is small, absent in many preparations, and easily declines during repeated stimulation, unlike that observed in the rabbit (Ueda *et al.*, 1981; Zhang *et al.*, 1984). It is very likely that the available pool of substance P is of small capacity and may be readily exhausted by repeated stimulation. The finding that only the first application of capsaicin elicited a contraction in the rat iris sphincter may also support this interpretation.

The physiological significance of substance P has been doubted in bovine iris sphincter (Suzuki & Kobayashi, 1983). Moreover, both canine and feline iris sphicter are insensitive to substance P (Kanda, Imaizumi & Watanabe; unpublished observation). Thus, among the mammalian iris sphincter muscles examined, rabbit iris is most susceptible to substance P. In accordance with these findings, motor function of the substance P-ergic nerve on iris sphincter muscle may be important physiologically in rabbit but not in the dog, cat, rat and guinea-pig (Kanda, Imaizumi & Watanabe; unpublished observation).

In a previous paper (Narita & Watanabe, 1981) a TTX-insensitive response to electrical stimulation was assumed to be a direct response of smooth muscle. In the present work its sensitivity to capsaicin pretreatment suggests that it may have been in part due to the direct release of substance P from nerve endings in the absence of action potentials. Similar TTX-resistant phenomena have been observed for the release of adenosine 5'-triphosphate from nerve endings in rabbit detrusor muscle (Chaudhry *et al.*, 1984), guinea-pig taenia coli and vas deferens (White *et al.*, 1981).

In conclusion, in the rat isolated iris sphincter muscle, the contractions induced by electrical stimulation are due mainly to the dominant cholinergic mechanism, but possibly also to a very small extent to substance P-ergic nerve stimulation, and the contractions produced by ACh and substance P, either applied exogenously or released from endogenous pools, are characterized by the pharmaco-mechanical coupling.

This work was supported by a grant (No. 59570952) from the Japanese Ministry of Education, Science and Culture.

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(Received January 29, 1985.
Revised March 15, 1985.
Accepted, April 22, 1985.)