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Mechanism of action of nicotine in isolated iris sphincter preparations of rabbit

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1 Nicotine produced a transient contraction of rabbit isolated iris sphincter muscle, a parasympathetic ganglion-free tissue. The response to nicotine was antagonized by hexamethonium, but was insensitive to tetrodotoxin (TTX). While single treatments with atropine, capsaicin or [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P (rpwwL-SP) partially blocked the response, combined treatment abolished it.

2 Chronic treatment of animals with nicotine added to the drinking water (about 12 mg kg^{-1} per day) had no effect on the responsiveness to nicotine or the pharmacological properties of nicotine-induced contraction.

3 These results suggest that acetylcholine and tachykinin(s) released via sodium channel-independent mechanisms from nerve terminals of parasympathetic and primary sensory nerves, respectively, are involved in the nicotine-induced contractile response.

Introduction

Nicotine is known to elicit a tetrodotoxin (TTX)-resistant sympathomimetic effect resulting from noradrenaline release in some adrenergically innervated isolated tissue preparations, such as rabbit pulmonary artery (Su & Bevan, 1970), rat vas deferens, cat splenic strip and guinea-pig atria (Jayasundar & Vohra, 1978), rabbit and guinea-pig aorta (Ikushima *et al.*, 1981; 1982). In these preparations, ganglion cells are absent. Since TTX blocks generation and propagation of sodium action potentials (Narahashi, 1974), these results are strong evidence that the site of these sympathomimetic actions of nicotine is the postganglionic nerve fibres or their nerve terminals (sodium action potential-independent mechanism) (Trendelenburg, 1965; Su & Bevan, 1970; Jayasundar & Vohra, 1978; Ikushima *et al.*, 1981; 1982).

On the other hand, the parasympathomimetic effect of nicotine on guinea-pig ileum and trachea was abolished by TTX. However, we have reported that nicotine also induces a TTX-resistant action, resulting from acetylcholine release, in isolated bronchi of rabbit, monkey and dog (Takayanagi *et al.*, 1984; Kizawa & Takayanagi, 1985a; Takayanagi *et al.*, 1988). Insensitivity to TTX suggests that, as with sympathetic postganglionic fibres, the site of parasympathomimetic action of nicotine is the postganglionic nerve terminals. Since parasympathetic ganglia are, however, commonly located within the target organs, involvement of the ganglion cells cannot be excluded completely.

In the present study, we investigated the mechanism of action of nicotine in an isolated iris sphincter preparation of rabbit, which, exceptionally, is densely innervated by parasympathetic neurones and free of ganglia (Schaeppi, 1966; Hasegawa *et al.*, 1987). Since it has recently been reported that there are capsaicin-sensitive primary sensory neurones in isolated preparations (Ueda *et al.*, 1981; 1982), the effect of nicotine on the sensory nerve terminals was also studied. Finally, as nicotine administered chronically may regulate the numbers of functional nicotinic receptors and/or receptors for mediators released by nicotine (Häggendal & Henning, 1980; Marks *et al.*, 1983; Yamanaka *et al.*, 1985; Kizawa *et al.*, 1988), the effects of chronic treatment with nicotine on the pharmacology of nicotine in rabbit iris sphincter were investigated.

Methods

Male albino rabbits weighing 2–3 kg were used. Each animal was allowed free access to food and tap water. They were kept in a temperature (24 ± 1°C)- and humidity (55 ± 5%)-controlled room with a 12 h light and dark cycle.

The methods used for experiments were essentially the same as those previously described (Hosoki *et al.*,

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1985; 1987). Eyeballs were removed and iris sphincters rapidly dissected in Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.54, $MgCl_2$ 1.20, KH_2PO_4 1.19, $NaHCO_3$ 25.0 and glucose 11.0. The sphincter was mounted in an organ bath which contained the solution bubbled with 95% O_2:5% CO_2. The temperature of the bath was maintained at 37°C. The preparation was equilibrated for about 90 min with an initial tension of about 1.5 mN; after spontaneous relaxation a resting tension of about 0.5 mN was maintained in the subsequent experimental protocol. The contractile responses to drugs were recorded isometrically. The tissue was exposed 3 to 5 times to carbachol 1 μM, and then usually 3 successive cumulative concentration-response curves to carbachol were determined. Thereafter, nicotine (0.1 mM) was applied and was washed out as soon as the contractile response had reached a maximum to avoid desensitization. The interval between exposures to nicotine was usually about 60 min. Under these conditions constant and reproducible contractions were observed at least 5 times.

In chronic nicotine treatment, rabbits (initial body weight 2.7 ± 0.1 kg, $n = 14$) drank either ordinary tap water or nicotine, 100 mg (as a base) l^{-1}, for 4 weeks (Yamanaka *et al.*, 1985; Kizawa *et al.*, 1988). The nicotine solution provided for drinking was freshly prepared daily by dissolving bitartrate salt in the drinking water.

Results are expressed as means ± standard errors with the numbers of experiments in parentheses. ANOVA was used to calculate statistical significance. A P value of <0.05 was considered a significant difference.

The following drugs were used: nicotine bitartrate (Nakarai), carbachol chloride, tris(hydroxymethyl)-aminomethane (Tris), capsaicin, atropine sulphate (Sigma), tetrodotoxin (Sankyo), hexamethonium dibromide (Tokyo Kasei), rpwwL-SP (Peninsula Labs.) and physostigmine salicylate (E. Merck). Flurbiprofen, prazosin, methysergide maleate and chlorpheniramine maleate were kindly donated by Kaken Pharmaceutical Co., Taito-Pfizer, Sandoz and Sankyo Co., respectively. Other chemicals were of analytical grade. Flurbiprofen (2 mM) was dissolved in 2% Na_2CO_3, Tris (20 mM) added and the pH brought to 8 by the addition of HCl. Capsaicin (10 mM) was dissolved in ethanol and rpwwL-SP (1 mM) was dissolved in deionized, distilled water. They were stored at −30°C.

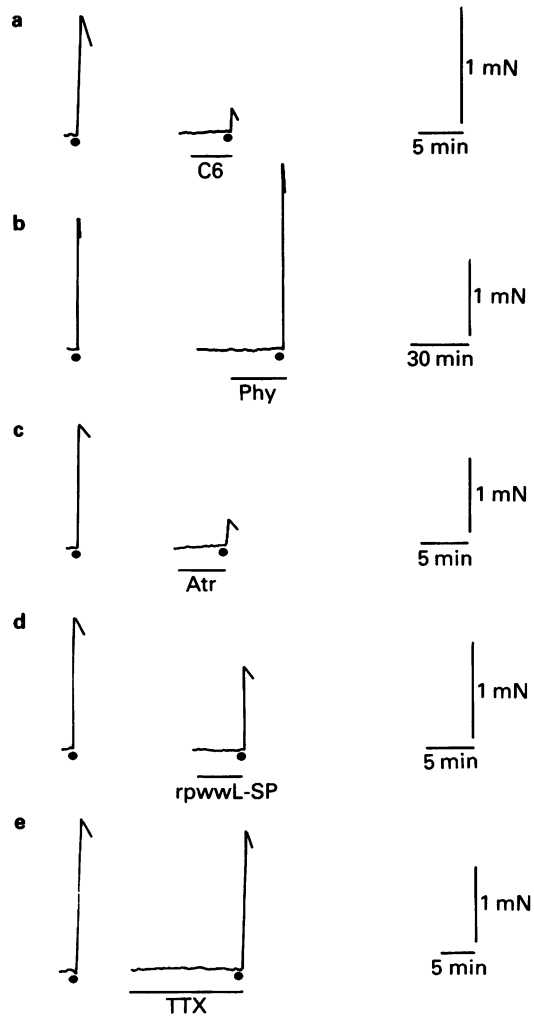

Figure 1 Typical tracings illustrating the effects of various drugs on the nicotine-induced contraction of rabbit isolated iris sphincter: (a) hexamethonium (C_6) (10 μM); (b) physostigmine (Phy) (0.1 μM); (c) atropine (Atr) (1 μM); (d) [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P (rpwwL-SP) (10 μM); (e) tetrodotoxin (TTX) (3 μM). Dots indicate application of 0.1 mM nicotine. Nicotine was washed out as soon as the contractile response had reached a maximum. Solid lines indicate the treatment with drugs. The preparation was treated for 15 min (TTX), 30 min (physostigmine) or 5 min (other drugs).

Results

Nicotine induced only a phasic contraction in the isolated sphincter muscle of rabbit (Figure 1). A concentration-response curve for nicotine was obtained at concentrations from 3 μM to 1 mM (Figure 2). The response to a nearly maximum concentration of nicotine (0.1 mM) was 1.56 ± 0.08 mN and 58.7 ± 1.8% of the maximum response to carbachol (30 μM: 2.75 ± 0.14 mN, $n = 83$) (Figure 2).

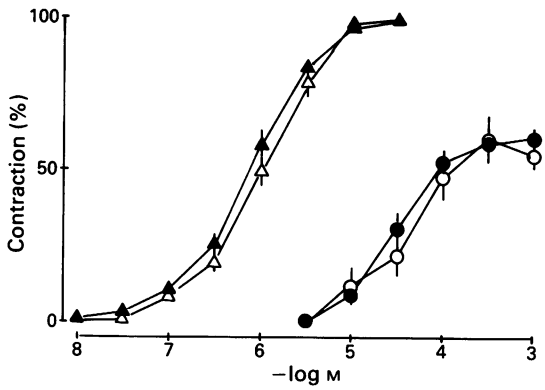

Figure 2 Concentration-response curves for nicotine (●, ○) and carbachol (▲, △) in the rabbit isolated iris sphincter from control (●, ▲) and chronically nicotine-treated (○, △) rabbits. Abscissa scale: negative log molar concentration of drugs. Ordinate scale: % of contraction induced by 30 μM carbachol of preparations obtained from control and treated rabbits. Each value is presented as a mean ± s.e.mean (vertical bar) of 6 experiments.

As shown in Table 1, the contractile response to 0.1 mM nicotine was blocked by hexamethonium (Figure 1a). The response was potentiated by physostigmine (1 μM) (Figure 1b), and reduced greatly, but not completely, by atropine (0.1 and 1 μM) (Figure 1c). The nicotine-induced contraction was also reduced to a limited extent by preceding repeated applications of capsaicin (10 μM), which releases and depletes tachykinins from trigeminal nerve endings (Gamse *et al.*, 1979; Theriault *et al.*, 1979) or a potent substance P antagonist rpwwL-SP (10 μM: Folkers *et al.*, 1984; Hosoki *et al.*, 1987; Muramatsu *et al.*, 1987) (Figure 1d). In the experiments using capsaicin, the drug was applied until the response disappeared (usually 3 to 5 times). As previously reported (Hosoki *et al.*, 1987), rpwwL-SP did not produce any contraction of rabbit iris sphincter. The difference between reductions of the nicotine-induced contraction by capsaicin and rpwwL-SP was not significant. Treatment of the capsaicin-treated preparations with atropine or simultaneous treatment with rpwwL-SP and atropine almost abolished the contractile response to nicotine.

However, the response to nicotine was not significantly influenced by TTX (3 μM) (Figure 1e), which was of sufficient concentration to abolish contraction to electrical field stimulation (Ueda *et al.*, 1981). The α_1-adrenoceptor antagonist prazosin (0.1 μM), 5-hydroxytryptamine (5-HT)-receptor antagonist methysergide (1 μM), H_1-histamine receptor antagonist chlorpheniramine and cyclo-oxygenase inhibitor flurbiprofen had no appreciable effect on the nicotine-induced contraction (Table 1).

During chronic nicotine treatment, each rabbit drank 11.50 ± 0.58 mg nicotine base kg^{-1} per day ($n = 14$). Following this treatment, concentration-response relationships for nicotine and carbachol were very similar to those obtained from control rabbits (Figure 2). In the iris sphincter muscle of

Table 1 Effects of drugs on the contractile responses to nicotine in isolated iris sphincter preparations of control rabbits

Treatment	*% of contraction*	n
Nicotine, 0.1 mM	100.0	
+ hexamethonium, 10 μM (5 min)	14.1 ± 2.3[a]	(6)
+ tetrodotoxin, 3 μM (15 min)	93.7 ± 1.4	(8)
+ atropine, 0.1 μM (5 min)	26.9 ± 2.9[a,b]	(7)
+ atropine, 1 μM (5 min)	18.4 ± 2.7[a,b]	(6)
+ physostigmine, 1 μM (30 min)	144.6 ± 3.2[a]	(6)
+ rpwwL-SP, 10 μM (5 min)	50.2 ± 11.9[a,c]	(6)
+ capsaicin, 10 μM	70.6 ± 4.7[a,c]	(15)
+ rpwwL-SP, 10 μM (5 min) & atropine, 1 μM (5 min)	2.0 ± 1.8[a]	(5)
+ capsaicin, 10 μM & atropine, 1 μM (5 min)	2.1 ± 0.6[a]	(6)
+ flurbiprofen, 1 μM (60 min)	90.8 ± 4.1	(6)
+ methysergide, 1 μM (5 min)	96.7 ± 1.4	(8)
+ prazosin, 0.1 μM (5 min)	91.7 ± 3.0	(8)
+ chlorpheniramine, 1 μM (5 min)	92.1 ± 4.2	(7)

[a] Significantly different from 100% at $P < 0.05$.
[b] Not significantly different from each other.
[c] Not significantly different from each other.
rpwwL-SP = [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P.

Table 2 Effects of drugs on the contractile responses to nicotine in isolated iris sphincter preparations of chronic nicotine-treated rabbits

Treatment	*% of contraction*	n
Nicotine, 0.1 mM	100.0	
+ hexamethonium, 10 μM (5 min)	6.8 ± 1.4[a]	(6)
+ tetrodotoxin, 3 μM (15 min)	91.4 ± 3.1	(7)
+ atropine, 0.1 μM (5 min)	20.2 ± 3.1[a,b]	(6)
+ atropine, 1 μM (5 min)	31.0 ± 4.2[a,b]	(5)
+ physostigmine, 1 μM (30 min)	134.8 ± 10.2[a]	(5)
+ rpwwL-SP, 10 μM (5 min)	42.2 ± 7.3[a,c]	(5)
+ capsaicin, 10 μM	64.2 ± 3.5[a,c]	(5)
+ rpwwL-SP, 10 μM (5 min) & atropine, 1 μM (5 min)	1.3 ± 0.9[a]	(5)
+ capsaicin, 10 μM & atropine, 1 μM (5 min)	1.5 ± 0.6[a]	(5)
+ flurbiprofen, 1 μM (60 min)	98.1 ± 3.4	(5)
+ methysergide, 1 μM (5 min)	101.6 ± 2.1	(5)
+ prazosin, 0.1 μM (5 min)	82.4 ± 8.2	(5)
+ chlorpheniramine, 1 μM (5 min)	93.5 ± 3.9	(5)

[a] Significantly different from 100% at $P < 0.05$.
[b] Not significantly different from each other.
[c] Not significantly different from each other.
rpwwL-SP = [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P.

chronic nicotine-treated rabbits, the response to nicotine (0.1 mM) was 1.40 ± 0.13 mN and 56.5 ± 3.7% of the maximum response to carbachol (30 μM: 2.56 ± 0.23 mN, $n = 44$); these values were not significantly different from corresponding control values. The pharmacological properties of the nicotine-induced contractions were not quantitatively different in preparations from control and chronically nicotine-treated animals (Table 1 *vs.* Table 2).

Discussion

Nicotine induced a contraction in the isolated iris sphincter preparation of the rabbit in this study. This contractile response was almost abolished by hexamethonium, suggesting that the effect of nicotine resulted from interaction with nicotinic receptors.

Since parasympathetic nerves synapse in the ciliary ganglion outside the eyeballs, the nicotinic receptors involved in isolated iris sphincter preparations are not those located on ganglion cell bodies, but those at postganglionic nerve fibres or nerve terminals. This is consistent with the total resistance of the nicotine action to TTX (3 μM), which abolishes contraction induced by electrical field stimulation in this tissue (Ueda *et al.*, 1981), and is in agreement with our previous assumption that in some preparations the parasympathomimetic response to nicotine may be produced mainly through a sodium channel-independent process (Takayanagi *et al.*, 1984; Kizawa & Takayanagi, 1985a,b; Kizawa *et al.*, 1988; Takayanagi *et al.*, 1988).

The nicotine-induced contraction was potentiated by physostigmine and reduced greatly, but not completely, by atropine, suggesting that the main part of the contraction was due to release of acetylcholine from parasympathetic nerve terminals. Prazosin, an α_1-adrenoceptor blocker, did not have any effect on the nicotine-induced contraction. Therefore, α_1-adrenoceptors do not participate in the response to nicotine in iris sphincter muscle, and the atropine-resistant contraction is non-cholinergic, non-adrenergic in nature. The present results suggest that the release of histamine, 5-HT and prostaglandins are not involved in the contractile mechanism of nicotine.

It is well known that capsaicin releases tachykinins from chemosensitive C-fibre afferents (Gamse *et al.*, 1979; Theriault *et al.*, 1979) of the ophthalmic trigeminal nerve in the eye (Ueda *et al.*, 1982). It has been recently reported that there are mRNAs of preprotachykinins A, precursors of substance P (SP) and neurokinin A (NKA) in the trigeminal ganglion (Nawa *et al.*, 1984), and that SP, NKA and neurokinin B (NKB), three mammalian tachykinins, are all present in rabbit iris sphincter (Taniguchi *et al.*, 1986). Repeated application of capsaicin causes a gradual tachyphylaxis to capsaicin, but not to carbachol or tachykinins (Hosoki *et al.*, 1987; Ueda *et al.*, 1981). Under such conditions, the nicotine-induced contraction was partially reduced and abol-

ished by further treatment with atropine. Similar results were obtained by use of rpwwL-SP, instead of repeated application of capsaicin. These results suggest that nicotine acts on nicotinic receptors located on the nerve terminals of the sensory nerves, and that the resulting release of tachykinin(s) is involved in the nicotine-induced non-cholinergic, non-adrenergic contraction.

In rabbit iris sphincter muscle, there coexist multiple receptor sites specific for SP, NKA and NKB, and each tachykinin interacts with the respective receptor sites to contract the iris sphincter smooth muscle (Hosoki *et al.*, 1985; 1987). Since rpwwL-SP antagonized the response to SP and NKA but not that to NKB (Hosoki *et al.*, 1987), SP or NKA, but not NKB, released by nicotine from sensory nerve terminals, may directly contract iris sphincter smooth muscle. Alternatively, tachykinin(s) may serve as neuromodulator(s) to regulate the release of an unknown mediator. Neither hypothesis can be excluded.

Chronic nicotine treatment has been shown to produce tolerance to acute behavioural effects and to induce an adaptive increase in density of nicotine binding sites in the brain (Marks *et al.*, 1983). Decreased sensitivity of peripheral adrenergic neurones and diminished responsiveness of detrusor muscle to nicotine have also been noted, respectively, in rats and rabbits receiving nicotine chronically (Häggendal & Henning, 1980; Kizawa *et al.*, 1988). The latter authors suggested that chronic nicotine treatment might cause a decrease in the number of nicotinic receptors and/or receptors for mediators released by nicotine (Kizawa *et al.*, 1988).

In the present study, chronic administration of nicotine by the same schedule as used for detrusor muscle studies (Kizawa *et al.*, 1988) had no effect on responsiveness to nicotine or on the pharmacological properties of nicotine action in iris sphincter. It may be that tolerance to nicotine develops in detrusor but not in ocular tissue, at least that from albino animals. Szüts *et al.* (1978) reported that intravenously administered [^{14}C]-nicotine is accumulated in the urinary bladder wall and in pigmented eyes, but not in non-pigmented eyes.

In summary, it is suggested that acetylcholine and tachykinin(s) released via sodium channel-independent mechanism(s) from nerve terminals of parasympathetic and trigeminal nerves, respectively, are involved in the nicotine-induced contractile response in the isolated iris sphincter preparation of rabbit. In other preparations where nicotine causes a TTX resistant parasympathomimetic action, such as bronchi of rabbit, monkey and dog (Takayanagi *et al.*, 1984; Kizawa & Takayanagi, 1985a; Kizawa *et al.*, 1988), it seems likely that the nicotinic receptors might also be located at the terminals of parasympathetic nerves.

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