Br. J. Pharmacol. (1993), **108**, 759–762 © Macmillan Press Ltd, 1993

Tachykinin receptors of the NK_2 type involved in the acetylcholine release by nicotine in guinea-pig bladder

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1 The effects of guanethidine and tachykinins on nicotine- and electrical stimulation-induced cholinoceptor responses were studied in isolated urinary bladder from the guinea-pig.

2 Acetylcholine release and the contractile response stimulated by nicotine were partially reduced by a sympathetic nerve blocker, guanethidine. Neurokinin A (but not substance P methyl ester or senktide) enhanced both acetylcholine release and contraction by nicotine in the presence of guanethidine.

3 Frequency-contraction curves (1 to 50 Hz) for electrical field stimulation (EFS) were partially reduced by atropine (1 μM), and after desensitization to α,β-methylene adenosine 5'-triphosphate, the atropine-resistant contraction to EFS was completely abolished. Guanethidine, the tachykinin antagonist [D-Arg[1], D-Pro[2], Trp[7,9], Leu[11]]-substance P and application of neurokinin A or substance P did not change the contractile response to EFS. Preganglionic nerve stimulation (5 Hz and 20 Hz) also evoked a similar response to EFS and was not influenced at all by guanethidine or neurokinin A.

4 We conclude that the ability of nicotine to release acetylcholine is enhanced both by endogenous tachykinins (probably released from sympathetic nerves) and by exogenously applied tachykinins as a result of interaction with NK_2 receptors in the urinary bladder.

Keywords: NK_2 tachykinin receptor; nicotine; acetylcholine release; sympathetic nerve; guinea-pig bladder

Introduction

We have shown that nicotine produces a tetrodotoxin-resistant contraction of isolated detrusor strips of guinea-pig, and have suggested that the drug might interact with the presynaptic nicotinic receptors located on parasympathetic cholinergic nerves and non-sympathetic purinergic nerves to release acetylcholine and a purine nucleotide, since the contractile response is blocked by a combination of atropine and desensitization of P_2-purinoceptors by α,β-methylene adenosine 5'-triphosphate (Hisayama *et al.*, 1988). The nicotine-induced acetylcholine output was partially reduced by guanethidine and [D-Arg[1], D-Pro[2], Trp[7,9], Leu[11]]-substance P (rpwwL-SP) to much the same extent. Pretreatment with guanethidine abolished the inhibitory effects of rpwwL-SP and *vice versa*. We therefore proposed that nicotine was also stimulating release of a tachykinin from sympathetic nerves and that acetylcholine release evoked by nicotine was enhanced by their action (Hisayama *et al.*, 1989). However, the possibility that tachykinins from an unknown source stimulate release of a mediator from sympathetic nerve endings, remained, although a sympathetic component is not observed in the contraction to electrical field stimulation (Ambache & Zar, 1970; Burnstock *et al.*, 1978; Fujii, 1988).

We have now determined the effects of exogenously applied tachykinins on the cholinoceptor responses induced by nicotine or electrical stimulation.

Methods

Female Hartley strain guinea-pigs, weighing between 300 and 500 g, were reared on a standard diet and given tap water to drink. The guinea-pigs were stunned by a blow on the head and exsanguinated from the femoral artery. The urinary bladder was rapidly removed and a longitudinal strip (about 2 mm × 15 mm) of the detrusor muscle was prepared. Each strip was suspended vertically under a resting load of 1 g in a 5 ml organ bath which contained Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.50, $MgSO_4$ 1.20, KH_2PO_4 1.20, $NaHCO_3$ 25.0 and glucose 10.0. The organ bath was maintained at 37°C and constantly gassed with carbogen (95% O_2 + 5% CO_2). The responses to drugs were recorded isotonically.

Electrical field stimulation was applied through a pair of platinum-wire electrodes by an electronic stimulator (Nihon Kohden, SEN-3301). Stimulus parameters were 0.5 ms duration, frequency of 1 to 50 Hz and supramaximal voltage for 2 s.

Preganglionic stimulation of the parasympathetic nerves innervating the bladder was performed as described by Hukovic *et al.* (1965). One ureter was dissected for about 20 mm from the bladder and thread was attached for passing the ureter through the electrodes; the detrusor strip was stimulated indirectly by rectangular pulses (50 V, duration 0.5 ms, 5 or 20 Hz for 2 s).

Desensitization to α,β-methylene adenosine 5'-triphosphate (α,β-MeATP) was produced by the method of Kasakov & Burnstock (1983).

When we determined the amount of acetylcholine released, each strip was incubated with 1 mM methanesulphonyl fluoride (MSF) for 30 min in order to inhibit acetylcholinesterase activity irreversibly. After priming 2 or 3 times with 300 nM carbachol, Krebs solutions containing various concentrations of drugs were superfused at a rate of 0.4 ml min^{-1} from the bottom and collected by aspiration at the top of an organ bath, and the effects of these drugs on acetylcholine output from the strips were evaluated. The superfusate was collected continuously on ice, divided into fractions for each 3 min period and assayed for acetylcholine as described by Shinkai *et al.* (1991).

Acetylcholine was determined by radioimmunoassay with rabbit antiserum raised against choline hemiglutarate-bovine serum albumin conjugates and tritiated acetylcholine with a specific activity of 86 Ci $mmol^{-1}$ according to the method of Kawashima *et al.* (1980, 1988). Assays were performed in duplicate at 4°C. To avoid overestimation by cross-reactivity with nicotine, the standard curve for acetylcholine was derived in the presence of nicotine as appropriate.

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A 200 μl portion of the superfusate was incubated overnight with 50 μl of the diluted antiserum (1:350) in Tris-HCl buffer (0.15 M, pH 7.4) containing 0.4% bovine gamma-globulin, 0.05% isofluorophosphate and 50 μl of tritiated acetylcholine (about 12.9 pg, 4200 c.p.m.). The same volume of superfusion fluid containing 0.1 mM nicotine served as a blank. Antibody-bound tritiated acetylcholine was separated from free by the ammonium sulphate method, and the radioactivity of the precipitates was quantified in a liquid scintillation counter.

Statistical analyses were performed by Student's t test. A P value of <0.05 was considered a significant difference.

Drugs used were nicotine bitartrate (Nakarai Chemicals, Ltd., Kyoto, Japan), atropine sulphate, α,β-MeATP, gamma-globulin (Sigma Chemical Co., MO, U.S.A.), hexamethonium dibromide (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), tetrodotoxin (Sankyo Co., Ltd., Tokyo, Japan), neurokinin A (NKA), substance P (SP), SP methyl ester (SPOMe), senktide, rpwwL-SP (Peninsula Laboratories, Inc., Belmont, CA, U.S.A.), acetyl[methyl-^{3}H]-choline chloride (Amersham Japan Co., Tokyo, Japan) and methanesulphonyl fluoride (Aldrich Chemical Co., Inc., U.S.A.). Guanethidine sulphate was donated by Ciba-Geigy (Japan), Ltd. (Hyogo, Japan). All drugs used were of analytical grade.

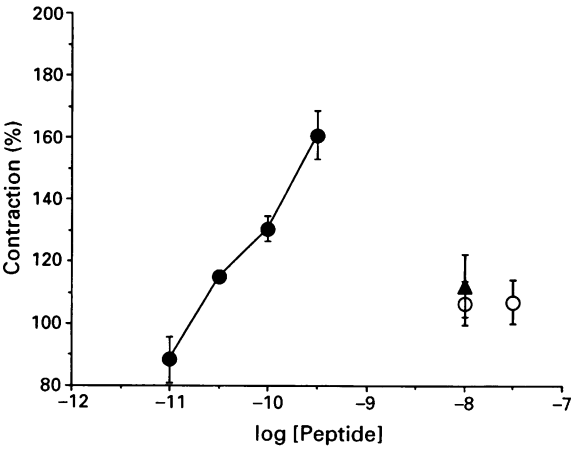

Figure 2 Effects of tachykinin receptor agonists on contractile responses induced by nicotine in the presence of guanethidine. Abscissa scale: log molar concentration of applied peptides. Ordinate scale: % of contraction induced by nicotine (0.1 mM) in the presence of guanethidine (3 μM): (●) neurokinin A; (○) senktide; (▲) substance P methyl ester (SPOMe). In the presence of guanethidine, nicotine-induced contraction was potentiated by neurokinin A but not by SPOMe or senktide.

Results

The effects of some drugs on acetylcholine release induced by nicotine (0.1 mM) are shown in Figure 1. The release evoked by nicotine was 74.2 ± 11.1 pg mg^{-1} tissue per 3 min ($n = 6$). The amount of acetylcholine released in the presence of test drugs is shown as % of that released to nicotine alone. The nicotine-induced acetylcholine output was greatly reduced by the sympathetic nerve blocker, guanethidine (3 μM) to 27.2 ± 6.5% ($n = 5$). The inhibitory effect of guanethidine was countered by the application of 1 nM neurokinin A (105.8 ± 12.5%, $n = 5$) but not the NK_1-selective agonist, SPOMe (22.3 ± 1.73%, $n = 5$) or the NK_3 selective agonist, senktide (27.7 ± 5.5%, $n = 5$). The same was true of the contractile response to nicotine (0.1 mM): in the presence of guanethidine, the nicotine-induced contraction was also potentiated by neurokinin A (30 μM to 0.3 nM) but not by SPOMe (10 nM) or senktide (10 nM) (Figure 2). In the presence of guanethidine, none of the tachykinins tested in this study at the concentration of up to 10 nM produced a contraction.

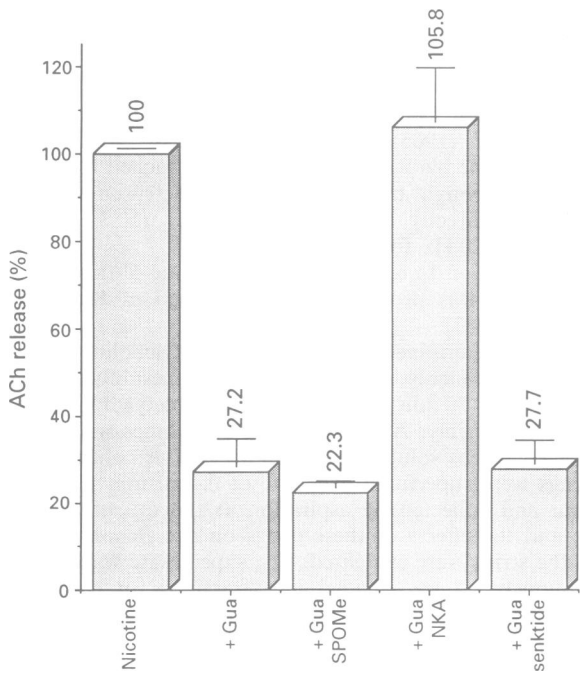

Figure 1 Effects of tachykinin receptor agonists on acetylcholine release evoked by nicotine from guinea-pig bladder. Gua: guanethidine 3 μM; NKA: neurokinin A 1 nM; SPOMe: substance P methyl ester 10 nM; senktide: senktide 10 nM. Nicotine-induced acetylcholine release was reduced by guanethidine to block modulator output from sympathetic nerves. Inhibitory effects of guanethidine were negated by neurokinin A but not by SPOMe or senktide.

Electrical field stimulation (EFS: frequency 1 to 50 Hz, duration 0.5 ms, trains 2 s, supramaximal voltage) produced tetrodotoxin-sensitive, transient contractions. The frequency-response curves were suppressed by atropine at all frequencies and the combined treatment of atropine and α,β-MeATP eliminated the nerve-mediated responses. The curves were not influenced by the treatment with guanethidine to block release of mediator from sympathetic nerve endings (Figure 3, Table 1).

Table 1 shows the effects of drugs on the contractions by EFS at 5 Hz and 20 Hz. Contractile responses to EFS were reduced by atropine and enhanced by physostigmine, indicating the existence of a cholinergic component. Hexamethonium, guanethidine, rpwwL-SP, neurokinin A, SPOMe and the metalloprotease inhibitor, phosphoramidon had no effect on the contraction caused by EFS.

Table 1 Effects of drugs on the contractile responses evoked by electrical field stimulation (EFS)

Treatment	*5 Hz*	*20 Hz*
Control	100	100
+ Hexamethonium, 100 μM, 5 min	102.3 ± 3.7	103.6 ± 3.9
+ Atropine, 1 μM, 5 min	51.9 ± 4.1*	61.0 ± 4.7*
+ Physostigmine, 0.1 μM, 30 min	136.0 ± 8.2*	146.7 ± 8.9*
+ Guanethidine, 3 μM, 30 min	106.7 ± 5.2	109.2 ± 5.1
+ rpwwL-SP, 10 μM, 5 min	113.2 ± 7.6	106.7 ± 5.3
+ Neurokinin A, 3 nM, 10 min	100.7 ± 6.1	104.5 ± 5.6
+ SPOMe, 10 μM, 10 min	95.6 ± 6.2	97.4 ± 2.8
+ Phosphoramidon, 10 μM, 20 min	105.6 ± 6.9	108.1 ± 6.2
+ Tetrodotoxin, 100 nM, 15 min	0.0 ± 0.0*	0.0 ± 0.0*

Each value shows mean ± s.e.mean of 6 experiments.
*Significant difference from control value (100%) at $P < 0.05$.
EFS (supramaximal voltage, duration 0.5 ms, 5 or 20 Hz for 2 s with 2 min intervals) was applied to the bladder. In contrast to results of nicotine-induced responses, contractions by EFS were not reduced by guanethidine and were not enhanced by neurokinin A.

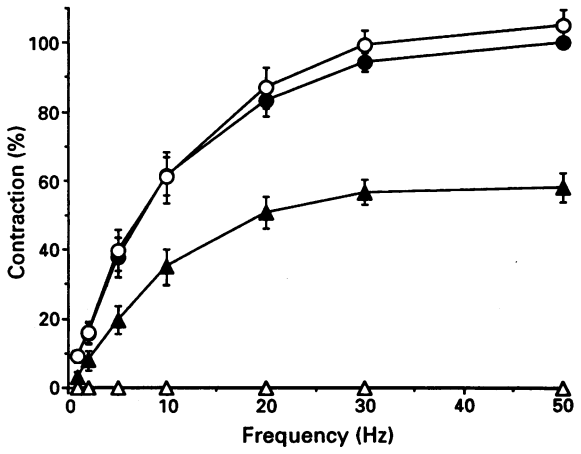

Figure 3 Effect of drugs on frequency-response curves in guinea-pig bladder. Abscissa scale: frequency. Ordinate scale: % of contraction induced by 50 Hz. (●) Control; (○) + guanethidine 3 μM; (▲) + atropine 1 μM; (△) + atropine after desensitization with α,β-methylene ATP 50 μM. Contractile responses to electrical field stimulation (EFS) were reduced by atropine and completely abolished by simultaneous treatment with atropine and α,β-methylene ATP. Though EFS induced a release of two excitatory transmitters, acetylcholine and a purine nucleotide as described in nicotine stimulation, guanethidine did not influence the curves.

When guinea-pig bladder preparations were stimulated indirectly through preganglionic nerves close to the ureter (5 Hz and 20 Hz), the evoked contractions were largely reduced by hexamethonium. Neurokinin A and guanethidine also had no effect on these contractile responses (Table 2).

Discussion

In this study, nicotine-induced acetylcholine output was reduced by guanethidine (Figure 1). Since chemical denervation with 6-hydroxydopamine abolished the inhibitory effect of guanethidine, and the drug did not inhibit the muscarinic receptor or purinoceptor mechanisms, it seems unlikely that the effect of guanethidine is non-specific (Hisayama *et al.*, 1988). We showed previously that nicotine contraction was not antagonized by bunazosin and yohimbine, and that exogenously applied noradrenaline did not cause a contraction even when the noradrenaline uptake mechanism and β-adrenoceptors were blocked (Hisayama *et al.*, 1988). The sympathomimetic effect of nicotine was non-adrenergic in nature, even if noradrenaline was released from the sympathetic nerve.

We previously determined that guanethidine and the tachykinin antagonist, rpwwL-SP, decreased the amount of acetylcholine released by nicotine to much the same degree. Pretreatment with guanethidine abolished the inhibitory effects of rpwwL-SP and *vice versa*. We proposed that acetylcholine release evoked by nicotine was increased by tachykinin released from sympathetic nerves (Hisayama *et al.*, 1989). But it is possible that tachykinins from an unknown site might have stimulated release of mediator from the sympathetic nerve endings. In this case, the excitatory effect of tachykinin (increasing acetylcholine output) would be abolished completely by a sympathetic nerve blocker.

Table 2 Effects of drugs on the contractile responses evoked by preganglionic stimulation

Treatment	*5 Hz*	*20 Hz*
Control	100	100
+ Hexamethonium, 100 μM, 5 min	53.9 ± 7.6*	62.9 ± 7.2*
+ Guanethidine, 3 μM, 30 min	106.6 ± 1.0	104.5 ± 8.9
+ Neurokinin A, 3 nM, 10 min	98.9 ± 6.6	101.5 ± 2.3
+ Tetrodotoxin, 100 nM, 15 min	0.0 ± 0.0*	0.0 ± 0.0*

*Significant difference from control value (100%) at $P<0.05$.
Each value shows mean ± s.e.mean of 5 experiments. Electrical stimulation (50 V, duration 0.5 ms, 5 or 20 Hz for 2 s with 2 min intervals) was applied to the supplying nerves (see Methods).

As shown in Figures 1 and 2, even in the presence of guanethidine which would block the release of any modulator from sympathetic nerves, neurokinin A enhanced acetylcholine output stimulated by nicotine. The possibility remains that nicotine could be releasing transmitters such as SP or neurokinin A from sensory nerve terminals. Densensitization to capsaicin, a drug that releases and depletes tachykinins from primary sensory neurones, had, however, no effect on the nicotine-induced contraction in guinea-pig bladder (Hisayama *et al.*, 1989). We may, therefore, reasonably conclude that sympathetic nerves are involved in the action of nicotine and are the site from which tachykinins are released.

As previously shown by Brading & Mostwin (1989) contractile responses to EFS were reduced by atropine and completely abolished by the stimultaneous treatment with α,β-MeATP and atropine (Figure 3). Since the response to nicotine is affected in the same way, it is likely that both methods of stimulation release the same excitatory transmitters. Two important differences were, however, noted between EFS and nicotine stimulation. Firstly, guanethidine had no effect on EFS-induced contraction at any frequency studied; this finding is in agreement with the accounts given by others (Ambache & Zar, 1970; Burnstock *et al.*, 1978; Fujii, 1988). One explanation for this lack of effect may be that modulator output from sympathetic nerves does not occur in response to EFS, but is initiated through presynaptic receptors and pharmacomechanical coupling. Secondly, neurokinin A (3 nM) did not enhance the contraction evoked by EFS (Table 1), although it clearly enhanced the ability of nicotine to release acetylcholine from nerve terminals. However, at this concentration, neurokinin A on its own has little contractile effect on the smooth muscle (Shinkai *et al.*, 1991). The metalloprotease inhibitor, phosphoramidon (10 μM) which potentiated pD_2 value and the maximal response of neurokinin A (unpublished observations), also had an influence on EFS-evoked responses.

Wessler *et al.* (1991) have suggested that transmural stimulation activates all excitable cell membranes, and because of this, presynaptic modulation cannot be observed using this technique. We therefore examined the response to presynaptic stimulation of vesical nerves as described by Hukovic *et al.* (1965); we found that hexamethonium largery reduced contractions evoked by presynaptic stimulation in contrast to its lack of effect on responses to EFS. Even with presynaptic stimulation, the responses are not influenced by guanethidine or neurokinin A (Table 2). The response to nerve stimulation is less susceptible to tachykinins than that to the nicotine stimulus.

Tachykinins facilitate the release of transmitter from the sympathetic nerves as a result of interaction with NK_2 receptors (Tousignant *et al.*, 1987; Regoli *et al.*, 1990). To determine the tachykinin receptor subtype involved in the acetylcholine output stimulated by nicotine, the effects of exogenous tachykinins in the presence of guanethidine to block the endogenous modulator release from sympathetic nerves were evaluated. Neither SPOMe nor senktide which were selective for NK_1 and NK_3 receptor subtypes, respectively, had any effect on the cholinoceptor responses in guinea-pig bladder. On the other hand, neurokinin A facilitated the acetylcholine output and cholinoceptor responses by nicotine at a concentration that had little effect on the smooth muscle.

In conclusion, the results presented confirm our previous suggestion that the nicotine-evoked release of acetylcholine from the bladder is enhanced by tachykinins simultaneously released from sympathetic nerves. The effect of the tachykinins is mediated by NK_2 receptors. These mechanisms do not appear to play a role in the response of normal bladder to excitatory nerve stimulation.

Our special thanks are due to Prof. Dr. Kawashima for providing us with the antiserum.

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(Received October 6, 1992
Accepted October 29, 1992)