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Prejunctional nicotinic receptors involved in facilitation of stimulation-evoked noradrenaline release from the vas deferens of the guinea-pig

†*L. Todorov, *K. Windisch, **H. Shersen, **A. Lajtha, †*M. Papasova & [1]*E.S. Vizi

*Institute of Experimental Medicine, Hungarian Academy of Sciences, 1450 Budapest, P.O.B. 67, Hungary; **Center of Neurochemistry, Ward's Island, New York 10035, U.S.A. and †Institute of Physiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

1 In guinea-pig prostatic vas deferens loaded with [^{3}H]-noradrenaline ([^{3}H]-NA), nicotinic receptor agonists, nicotine and dimethylphenylpiperazinium (DMPP) enhanced the resting and facilitated the stimulation-evoked release of [^{3}H]-NA in a concentration-dependent fashion. The effect of nicotine on both contraction of vas deferens and release of NA in response to field stimulation was stereospecific in favour of the naturally occurring (−)-enantiomer. Prolonged (15 min) exposure to (−)-nicotine resulted in a cessation of the facilitatory effect on NA release and on responses of the vas deferens to field stimulation.

2 The rank order of agonist potency in facilitating NA release was DMPP = (−)-nicotine > (+)-nicotine. Cytisine had no agonistic activity. The dissociation constants (K_D) of antagonists were 9.3 ± 0.6 and 31.4 ± 2.4 μM for (+)-tubocurarine and hexamethonium, respectively, when (−)-nicotine was used as agonist. α-Bungarotoxin had no antagonistic activity. These findings suggest that nicotinic receptors located on noradrenergic axon terminals are different from those located postsynaptically in striated muscle or ganglia but seem similar to those present on cholinergic axon terminals at the neuromuscular junction.

3 Cotinine, the breakdown product of nicotine failed to have any agonistic activity indicating that nicotine itself is responsible for the effects observed on axon terminals.

4 Stimulation of presynaptic muscarinic receptors by oxotremorine prevented the nicotine-induced facilitation of [^{3}H]-NA release, indicating the presence of both inhibitory muscarinic and facilitatory nicotinic receptors on noradrenergic axon terminals.

Introduction

A well-established property of nicotine in the peripheral nervous system is its agonist activity at nicotinic cholinoceptors in autonomic ganglia which give rise to the release of noradrenaline and acetylcholine from postganglionic sympathetic and parasympathetic axon terminals, respectively. In addition, there is universal agreement (cf. Westfall *et al.*, 1987) that nicotinic receptor stimulation of the nerve terminals of the nigrostriatal pathway enhances the resting release of dopamine from both synaptosomal (cf. de Belleroche & Bradford, 1978; Rapier *et al.*, 1988) and slice preparations (Westfall, 1974; Giorgueieff *et al.*, 1976). The effect of nicotinic receptor agonists on stimulation-evoked release of transmitters from axon terminals is rather controversial. While nicotine and other nicotinic receptor agonists have no effect on noradrenaline (NA) release from rat heart (Fuder *et al.*, 1982) and brain vessels (Edvinsson *et al.*, 1977), evidence is available that activation of presynaptic nicotinic receptors results in a facilitation of stimulation-evoked transmitter release in guinea-pig heart (NA, Lindmar *et al.*, 1968; Westfall & Brasted, 1972) and in skeletal muscle (acetylcholine, Vizi *et al.*, 1987; Vizi & Somogyi, 1989).

The purpose of the present investigation was to determine whether nicotine itself releases NA and/or facilitates electrical stimulation-evoked release. In addition, an attempt was made to study the mechanism of this action by examining the effects of pharmacological agents known to influence the action of nicotine on both contractions of, and [^{3}H]-NA release from, guinea-pig isolated vas deferens in response to field stimulation.

[1] Author for correspondence.

Methods

Guinea-pigs of either sex, weighing 300–500 g, were killed by a blow to the head under light ether anaesthesia. The excised vas deferens was incubated for 40 min in Krebs solution (composition in mM: NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, Mg_2SO_4 1.2, $NaHCO_3$ 25.0 and glucose, 11.5) at 37°C containing [1-7,8-^{3}H]-noradrenaline (490 kBq ml^{-1}, 555 GBq mmol^{-1} sp. act., Amersham). During the incubation, the medium was bubbled with 95% O_2 and 5% CO_2. After incubation the vasa were washed several times with Krebs solution and suspended in 2.5 ml organ baths. The preparations were superfused (1 ml min^{-1}) for 90 min with Krebs solution at 37°C containing ascorbic acid (3×10^{-3} M) Na_2EDTA (10^{-4} M) and prednisolone (10^{-4} M) and the effluent was discarded. Subsequently, 3 min fractions were collected. The total radioactivity of tritiated compounds ([^{3}H]-NA and ^{3}H-metabolites) released from the preparations was monitored by adding 1 ml samples of the perfusate to 7 ml of liquid scintillation fluid prepared by mixing 6 g 1,4-*bis*-(5-phenyloxazolyl-2)-benzene (POPOP), 2 g of 2,5-diphenyloxazole (PPO), 2000 ml toluene, and 100 ml of Triton X-100. Radioactivity was determined in a liquid scintillation counter (Packard 544) and the counts were converted to absolute activity by the external standard method. Release of tritium was expressed in Bq g^{-1} and as a percentage of the amount of radioactivity in the tissue at the time when the sample was collected (fractional release). A computer programme was used for calculation of fractional release. For assay of residual radioactivity, the tissues were blotted with filter paper, weighed, homogenized in 1 ml of ice-cold 10% trichloroacetic acid, and centrifuged at 1500 *g* for 10 min. An aliquot of the supernatant was assayed for radioactivity.

The tissues were stimulated through platinum ring electrodes, one located above and the other below the suspended vas deferens. Supramaximal ($>10\,V\,cm^{-1}$) field stimuli of 0.5 ms duration were applied at 8 Hz, for 1 min or 20 s periods by means of an Eltron (Budapest, Hungary) stimulator. Drugs were added to the organ bath 30 s before the second stimulation unless stated otherwise.

In some experiments contraction was measured with a force displacement transducer and recorded on a potentiometric recorder (Goerz Servogor). The EC_{50} value (concentration needed to enhance the response by 50%), was calculated from concentration-response curves. S_1 and S_2 refer to first and second periods of stimulation, respectively.

To determine the proportion of the released radioactivity attributable to [^{3}H]-NA the perfusate was analysed by high performance liquid chromatography (h.p.l.c.) combined with radiochemical detection (Vizi *et al.*, 1985). In agreement with others (Roffler-Tarlov & Langer, 1971), the release of radioactivity in response to electrical stimulation alone or with nicotine receptor stimulation was due largely to [^{3}H]-NA.

In some experiments the apparent equilibrium dissociation constant (K_D) for antagonists was determined by the dose-ratio method described by Furchgott (1972). The following equation was used to relate the dissociation constant (K_D) to the dose-ratio and the antagonist concentration

$$K_D = \frac{a}{DR - 1}$$

Where DR is the concentration-ratio, i.e. the EC_{50} value for agonist (nicotine) in the presence of the antagonist divided by the EC_{50} value in the absence of antagonist and a is the concentration of antagonist. EC_{50} indicates the concentration of agonist needed to produce a 50% increase of S_2/S_1 value. Three different concentrations of agonists were used to establish a concentration-response curve and each point was derived from three different experiments.

Statistics

Statistical analysis was carried out with one way analysis of variance followed by Dunn test. All data in the text are expressed as means ± s.e.mean.

Drugs

The following drugs were used: (−)-[7,8-^{3}H]-noradrenaline (Amersham); (−)-nicotine hydrogen tartrate (Sigma); (+)-nicotine hydrogen tartrate (Sigma); cytisine (Sigma); (−)-cotinine (Sigma); oxotremorine (Loba Chemie, Vienna); dimethylphenylpiperazinium (DMPP, Sigma); hexamethonium bromide (Sigma); (+)-tubocurarine (Wellcome); cocaine hydrochloride (Chinoin, Hungary); desipramine (EGIS, Hungary), prazosin hydrochloride (Bayer); prednisolone (Di-Adreson, Organon); α-bungarotoxin (Sigma).

Results

After 40 min loading with [^{3}H]-NA, followed by a 90 min washout, the tissues contained 3,850,000 ± 152,500 Bq g^{-1} ($n = 16$) radioactivity. By use of h.p.l.c. combined with radiochemical detection it was established that 87.5 ± 4.2% of this was [^{3}H]-NA.

At rest, during the 3 min collection periods 0.70 ± 0.05% (25,500 ± 1082 Bq g^{-1}) ($n = 16$) of the total content of radioactivity was released. In response to field stimulation 1.16 ± 0.06% of radioactivity present in the tissue at the time of stimulation, was released over basal release ($n = 24$). The stimulation-evoked release was fairly constant: the ratio (S_2/S_1) between the fractional amounts of radioactivity released by two consecutive stimulations (27 min elapsed between the two stimulations) was 0.98 ± 0.04 ($n = 26$). During the experiments only 11.60 ± 0.50% ($n = 56$) of the total radioactivity taken up and stored was released.

Effect on resting release of [^{3}H]-noradrenaline

When the tissue was exposed for 1 min to (−)-nicotine (50 μM) or DMPP (50 μM) the resting release of radioactivity was transiently enhanced by 16,200 ± 1150 ($n = 4$) and 18,150 ± 1330 Bq g^{-1} ($n = 4$) respectively, representing 0.41 ± 0.03% and 0.47 ± 0.04% of tissue radioactivity content. When the exposure time was longer (5 min) the total amount of radioactivity released was not further increased. (−)-Nicotine (10 μM), DMPP (10 μM) or cytisine (100 μM) had no effect on resting release. Of the radioactivity released by nicotinic receptor stimulation, 71.5 ± 8.3% was [^{3}H]-NA. (+)-Tubocurarine (100 μM) completely prevented the effect of (−)-nicotine (50 μM) on resting release of [^{3}H]-NA.

Effect on stimulation-evoked release of [^{3}H]-noradrenaline

(−)-Nicotine or DMPP enhanced stimulation-evoked release of radioactivity in a concentration-dependent manner (Table 1). However (+)-nicotine was 38 times less effective than (−)-nicotine and cytisine, (10–100 μM), a compound with high affinity for nicotinic binding sites, failed to affect the release. The nicotine metabolite, cotinine (Jacob *et al.*, 1988) even in 100 μM concentration did not facilitate the release.

Prolonged exposure (15 min) to (−)-nicotine (50 μM) resulted in a cessation of the facilitatory effect on the stimulation-evoked release of [^{3}H]-NA (Table 2).

Nicotinic receptor antagonists (α-bungarotoxin hexamethonium or (+)-tubocurarine) alone did not affect the S_2/S_1 ratio for [^{3}H]-NA release (Table 3). However, hexamethonium and (+)-tubocurarine, but not α-bungarotoxin, prevented or reduced the effect of (−)-nicotine or DMPP on the stimulation-evoked release (Table 3). K_D values were calculated to be 9.3 ± 0.6 μM and 31.4 ± 2.4 μM for (+)-tubocurarine and hexamethonium, respectively, when (−)-nicotine was the agonist, and 41.8 ± 1.8 and 60.0 ± 0.6 respectively when DMPP was the agonist.

Oxotremorine decreased [^{3}H]-NA release in an atropine-sensitive manner, and completely inhibited the facilitatory effect of (−)-nicotine on stimulation-evoked release of [^{3}H]-

Table 1 Effect of nicotinic receptor agonists on stimulation-evoked release of [^{3}H]-noradrenaline ([^{3}H]-NA)

Drugs		*[^{3}H]-NA release* S_2/S_1	
Control		0.98 ± 0.04	(26)
(−)-Nicotine	1 μM	0.97 ± 0.06	(4)
(−)-Nicotine	2 μM	1.19 ± 0.02*	(4)
(−)-Nicotine	10 μM	1.72 ± 0.20*	(4)
(−)-Nicotine	50 μM	2.85 ± 0.45*	(4)
(−)-Nicotine	100 μM	3.12 ± 0.08*	(4)
(+)-Nicotine	100 μM	0.89 ± 0.06	(3)
(+)-Nicotine	200 μM	0.96 ± 0.07	(3)
(+)-Nicotine	500 μM	1.63 ± 0.11*	(3)
DMPP	1 μM	1.03 ± 0.07	(4)
DMPP	10 μM	1.57 ± 0.02*	(4)
DMPP	50 μM	2.97 ± 0.56*	(4)
Cytisine	100 μM	1.07 ± 0.08	(3)
Cotinine	100 μM	0.94 ± 0.05	(5)

Twenty seven minutes elapsed between the two stimulations (8 Hz, 480 shocks). Drugs were added to the organ bath 30 s prior to second stimulation (S_2). Note that rank order of activity of nicotinic agonists: DMPP = (−)-nicotine > (+)-nicotine ≫ cytisine. Mean ± s.e.mean. Number of experiments is in parentheses. The EC_{50} for (−)-nicotine and (+)-nicotine was 10.2 and 410 μM, respectively.
* Significant difference from control, $P < 0.05$.
DMPP = dimethylphenyl piperazinium.

Table 2 Effect of prolonged exposure to (−)-nicotine on [^{3}H]-noradrenaline release

	Control	(−)-Nicotine[1] (50 μM) 30 s exposure	15 min exposure
S_2/S_1	0.97 ± 0.07 (4)	2.48 ± 0.12* (4)	0.99 ± 0.12 (4)

[1] (−)-Nicotine was added to the organ bath 30 s or 15 min before the second stimulation (S_2). * Significant difference from control, $P < 0.01$. Twenty seven minutes elapsed between the two stimulations (8 Hz, 480 shocks).

NA (Table 4). Oxotremorine failed to affect the enhanced resting release induced by (−)-nicotine (50 μM).

Effect on mechanical responses of vas deferens to field stimulation

The response to field stimulation is biphasic: an initial fast contraction is followed by a slow response (Figures 1 and 2, left side). While (−)-nicotine (20 μM) produced a transient contraction and potentiated both phases of the response to stimulation, (+)-nicotine was much less effective (Figure 2 middle). Prazosin mainly reduced the second phase and slightly affected the initial phase of the muscle response to stimulation but prevented the transient contraction produced by (−)-nicotine (50 μM) (Figures 1 and 2, right). If the 1 min nicotine applications were repeated at intervals no shorter than 30 min, contractions and potentiation of responses to stimulation were reproducible. The EC_{50} value for (−)-nicotine potentiating the slow, NA-mediated response was 11 μM ($n = 4$).

Table 3 Hexamethonium and (+)-tubocurarine prevent the effect of nicotinic receptor agonists on [^{3}H]-noradrenaline ([^{3}H]-NA) release

Drugs		*[^{3}H]-NA release* S_2/S_1	
Control		0.97 ± 0.06	(6)
Hexamethonium,	300 μM	0.89 ± 0.08	(3)
(−)-Nicotine	50 μM	3.01 ± 0.21*	(6)
DMPP	50 μM	3.21 ± 0.21*	(3)
Hexamethonium, + (−)-nicotine	300 μM 50 μM	1.12 ± 0.10	(3)
(+)-Tubocurarine,	100 μM	0.84 ± 0.12	(3)
(+)-Tubocurarine, + (−)-nicotine	100 μM 50 μM	1.02 ± 0.22	(4)
(+)-Tubocurarine, + DMPP	100 μM 50 μM	1.55 ± 0.22*	(4)
α-Bungarotoxin,	3 μg ml^{-1}	1.01 ± 0.04	(4)
α-Bungarotoxin, + (−)-nicotine	3 μg ml^{-1} 50 μM	2.75 ± 0.15*	(4)

Twenty seven minutes elapsed between the two stimulations (8 Hz, 480 shocks).
Antagonists were added into the perfusion fluid 15 min prior to second stimulation. (−)-Nicotine or DMPP were added 30 s prior to second stimulation. Number of experiments is in parentheses. * Significant difference from control, $P < 0.05$.
DMPP = dimethylphenylpiperazinium.

Table 4 Interaction between muscarinic inhibitory and nicotinic stimulatory action on stimulation-evoked release of [^{3}H]-noradrenaline ([^{3}H]-NA)

	Drugs		*[^{3}H]-NA release* S_2/S_1		*Signif. $P < 0.05$*
1	Control		0.95 ± 0.06	(6)	
2	(−)-Nicotine,	50 μM	2.91 ± 0.18	(4)	2:1
3	Oxotremorine,	1 μM	0.54 ± 0.03	(4)	3:1
4	Oxotremorine, + (−)-nicotine,	1 μM 50 μM	1.04 ± 0.09	(4)	4:2 4:3
5	Atropine	1 μM	1.02 ± 0.08	(4)	
6	Atropine + oxotremorine, + (−)-nicotine,	1 μM 1 μM 50 μM	3.50 ± 0.21	(4)	6:4

Twenty seven minutes elapsed between the two stimulations (8 Hz, 480 shocks).
(−)-Nicotine and DMPP were added 30 s prior to second stimulation. Atropine and oxotremorine were added 15 min prior to second stimulation. Significance is calculated between groups as indicated in final column. Number of experiments in parentheses.
DMPP = dimethylphenylpiperazinium.

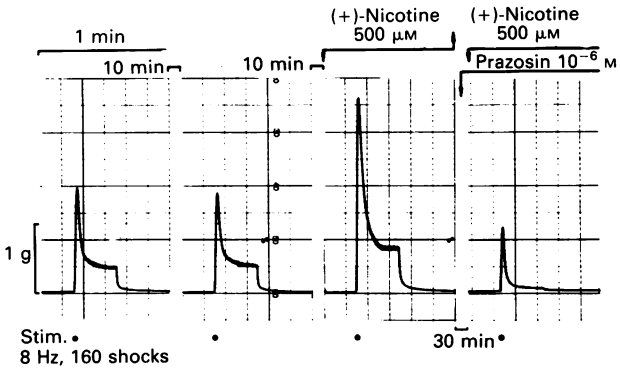

Figure 1 Effect of (−)-nicotine on contraction of prostatic part of guinea-pig vas deferens in response to stimulation. Field stimulations as indicated. Intervals between stimulation 10 and 30 min as indicated. Note that (−)-nicotine (20 μM) potentiated both phases of contraction in response to stimulation. The transient contraction and the second phase were mainly prazosin-sensitive.

Discussion

The presence of prejunctional nicotinic receptors on noradrenergic axon terminals in vas deferens of the guinea-pig is supported by our observations that the nicotinic receptor agonists, (−)-nicotine or DMPP, facilitated both the resting and stimulation-evoked release of [^{3}H]-NA and that these facilitations of release were reduced by hexamethonium or (+)-tubocurarine. It is unlikely that the elevated S_2/S_1 ratio in the presence of nicotine or DMPP was due simply to an additive effect of the increase in basal overflow to the release due to electrical stimulation. Thus, (−)-nicotine or DMPP at a

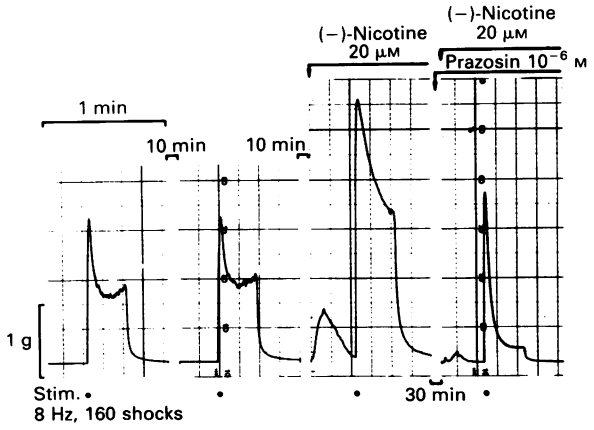

Figure 2 Effect of (+)-nicotine on contraction of prostatic part of guinea-pig vas deferens in response to stimulation. Field stimulation was indicated. Intervals between stimulations 10 and 30 min as indicated. Note that (+)-nicotine (500 μM) also potentiated the contraction, but in much higher concentrations than (−)-nicotine.

concentration (10 μM) that did not affect resting release, significantly enhanced stimulation-evoked release. Furthermore, when the transient contraction to (−)-nicotine had ceased the contraction of the muscle in response to stimulation was still potentiated (Figure 1).

The effect of nicotine on both contraction of vas deferens and release of NA in response to field stimulation was stereospecific with (+)-nicotine being 40 times less effective in facilitating NA release than (−)-nicotine. A similar observation was made by Rapier *et al.* (1988) in measuring dopamine release from striatal synaptosomes. The rank order of potency for presynaptic agonistic activity in our experiments was DMPP = (−)-nicotine > (+)-nicotine ≫ cytisine, whereas that obtained by Wonnacott (1987) using rat brain with binding techniques was cytisine > (−)-nicotine > DMPP > (+)-nicotine. Our observation for the rank order of antagonists ((+)-tubocurarine > hexamethonium > α-bungarotoxin) indicates that the presynaptic nicotinic receptors located on the axon terminals of sympathetic neurones are different from the well-characterized receptors located somatodendritically (e.g., in the ganglion, where the rank potency order of antagonists is hexamethonium ≫ (+)-tubocurarine), (Paton & Zaimis, 1952) or postjunctionally on skeletal muscle (the rank order: (+)-tubocurarine > α-bungarotoxin > hexamethonium), but are similar to those located on cholinergic axon terminals at the neuromuscular junction (Vizi *et al.*, 1987; Vizi & Somogyi, 1989). The nicotinic receptors of electric organs of the skate and eel are different from those of muscle and ganglia (Loring & Zigmond, 1988). The existence of multiple nicotinic receptors is supported by recent molecular biology studies in which different subtypes of nicotinic receptor were identified in brain (Goldman *et al.*, 1987; Colquhoun *et al.*, 1987).

The second, tonic part of the motor response to field stimulation of this preparation is noradrenergic (Bentley & Sabine, 1963; Vizi & Burnstock, 1988) and is mediated via α_1-adrenoceptors (Figure 1). The first phasic contraction was also potentiated by (−)-nicotine and the extent of potentiation of the first rapid twitch and the tonic second contraction was similar (Figures 1 and 2).

For guinea-pig vas deferens it was suggested (Birmingham & Wilson, 1963; Robinson, 1969; Fukushi & Wakui, 1987) that cholinergic input exerts a dual modulatory effect on sympathetic neuro-effector transmission: through presynaptic muscarinic receptors acetylcholine (ACh) would reduce the release of NA, whereas, on the effector cells, also through muscarinic receptors, ACh would enhance the response (Stjärne, 1975). In our study oxotremorine, a muscarinic receptor agonist, reduced the release of [^{3}H]-NA and antagonized the nicotinic receptor-mediated facilitation of [^{3}H]-NA release evoked by field stimulation. Thus if muscarinic receptors are stimulated, the nicotinic receptor-mediated potentiation of NA release is not operative. But the fact that both muscarinic and nicotinic receptors are involved in the modulation of NA release indicates that the cholinergic innervation of the vas deferens (Birmingham & Wilson, 1963; Knoll *et al.*, 1972) may play a role in presynaptic modulation of NA release from the sympathetic axon terminals. The concentration of nicotine achieved during smoking (0.3–2 μM, Russell *et al.*, 1980; Jacob *et al.*, 1988) is comparable to that which affects prejunctional nicotinic receptors in the guinea-pig isolated vas deferens.

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