

Effects Of The Nicotine On The Neurogenic Contractile Responses via Nicotinic Acetylcholine Receptors In Rabbit Bladder Tissue: Reactive Oxygen Species Do Not Plays a Role In This Mechanism

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SUMMARY

The aim of the study was to determine whether the electrical field stimulation (EFS)-evoked contractions are altered in rabbit bladder strips in the presence of nicotine and if an alternation occurs to investigate the effects of reactive oxygen species (ROS) on nicotine-induced alternation in isolated rabbit bladder. EFS-evoked contractile responses were recorded from bladder strips obtained from rabbits with an isometric force displacement transducers. Effects of nicotine on EFS evoked contractions were examined. The effects of hexamethonium, cadmium (Cd^{2+}), allopurinol and tiron were tested on the EFS-evoked contractions in the presence of nicotine. Nicotine led to increase in the amplitudes of the EFS-evoked contractile responses in a dose dependent manner. Cd^{2+} and hexamethonium inhibited nicotine-induced increase in EFS-evoked responses whereas allopurinol and tiron had no effect. Nicotine increased the EFS-evoked contractile responses possibly by facilitating neurotransmitters release from nerve terminals by a mechanism dependent on the influx of Ca^{2+} from voltage gated Ca^{2+} channels (VGCC) via activation of nAChRs in isolated rabbit bladder. ROS does not play a physiological role on the regulation of these neurotransmitter release.

Key Words: Nicotine, rabbit bladder, nicotinic acetylcholine receptors (nAChRs), electrical field stimulation, reactive oxygen species.

Introduction

Nicotine is the alkaloid isolated from the leaves of the tobacco plant. Nicotine act as an agonist of nicotinic acetylcholine receptors (nAChRs), which are belong to a superfamily of ligand gated ion channels and have seventeen subunits identified at the present. Nicotinic receptors are located in both central and peripheral nervous system (1-3). Nicotine modulates the neurotransmitter releases and nAChRs play key roles in synaptic transmission but the mechanisms underlying this action poorly understood. Previously in various isolated tissues such as guinea pig vas deferens, rabbit pulmonary artery and rat stomach different it was reported that nicotine increases the release of neurotransmitters following nerve stimulation via nAChRs in guinea-pig hippocampus (3-5). In an aganglionic vas deferens it was shown

that presynaptic nAChRs modulates acetylcholine release (6), similarly acetylcholine release modulated presynaptically negatively by muscarinic agonists and positively by nicotinic agonists in guinea pig ileum myenteric plexus (7). Different research group demonstrated that activation of ganglionic nicotinic receptors localized at the celiac ganglia induces noradrenalin releases from the rat stomach (5).

Effects of reactive oxygen species (ROS) on synaptic neurotransmission was investigated by different research groups previously (9-11). It was indicated that ROS might inhibit neurotransmitter release by decreasing Ca^{2+} entry via presynaptic Ca^{2+} channels (9). Also in an other study it was demonstrated that endogenous H_2O_2 production by Ca^{2+} dependent mechanisms could modulate vesicular neurotransmitter releases (10).

The aim of the present study was to investigate the effect of nicotine on the electrical field stimulation (EFS)-evoked contractile responses and if an alternation occurs to investigate the effects of ROS on nicotine-induced alternation in isolated rabbit bladder.

Methods

Animals:

A total of 20 New Zealand white rabbits weighing 2.5 to 3 kg were used for the experiments. All animals were kept under controlled temperature (23.2°C) and humidity (55.5%) with a 14-hour light and 10-hour dark cycle. They were fed Standard lab chow and given tap water. Experiments were performed in agreement with the ethical standards in Helsinki Declaration and this study was approved by Gazi University Ethics Committee (project number: G.U.ET-05.073) for Animals. Animals were killed by exsanguination and the bladder was rapidly excised, opened lengthwise, emptied. Adherent fat, gross connective tissues were removed and uniform longitudinal strips (20mm×3mm×natural thickness) were prepared.

Organ Chamber Experiments:

Each strip was mounted isometrically at 1 g resting tension in the organ bath containing 15 ml Krebs-Henseleit solution (composition in millimoles per liter: NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 1.3, $MgCl_2 \cdot 6H_2O$ 0.5, $Na_2HPO_4 \cdot 2H_2O$ 0.9, $NaHCO_3$ 24.9, glucose monohidrat 11). The pH of the solution was 7.4 after being bubbled with the gas mixture of 95% O_2 and 5% CO_2 and the solution was maintained at 37°C. Tissues were allowed to equilibrate for at least 1 hour prior to experimental procedures. Isometric contractions were evoked by EFS through a pair of platinum electrodes, with 8 Hz of stimulation frequency with 10 sec trains of impulses delivered every 2 min. Pulses of 1 ms duration with a voltage of 60 V were delivered by a stimulator (STPT 03, May Research stimulator; COMMAT Iletisim Co. Ankara, Turkey). EFS-evoked responses were recorded via isometric force displacement transducers (FDT 10-A, May IOBS 99, COMMAT Iletisim Co. Ankara, Turkey) connected to an online computer via 4-channel transducer data acquisition system (MP30B-CE, BIOPAC Systems Inc, Santa Barbara, CA) using software (BSL PRO v 3.6.7, BIOPAC Systems Inc, Santa Barbara, CA) which also had the capacity to analyse the data. Following 30 min after the EFS-evoked responses reached steady state, to test the contribution of purinergic and cholinergic components to the EFS-evoked responses, the tissue was treated with α, β -methylene ATP (10^{-5} M) a purinergic agonist that desensitise

purinergic receptors and a muscarinic receptor blocker, atropine (10^{-6} M). Effects of tetrodotoxin (TTX) (3×10^{-6} M) and hexamethonium (10^{-5} M) were also tested on the EFS-evoked responses. To test the effects of nicotine at varying concentrations (10^{-6} M, 3×10^{-6} M, 10^{-5} M, 3×10^{-5} M) on the EFS induced contractile responses nicotine was applied to preparations. After washing the preparations four times every ten minutes for 30 min, EFS was delivered and the same experimental procedure was repeated in the same tissue in the presence of hexamethonium (10^{-5} M), cadmium (Cd^{2+}) (10^{-4} M), allopurinol (10^{-6} M) or tiron (3×10^{-5} M).

In on other set of experiments, effect of nicotine was examined on the preparations in the organ bath at 1 g resting tension. Then, to test the relaxation effect of nicotine, nicotine was applied to preparations which were precontracted with histamine (3×10^{-5} M). EFS was not applied to these preparations.

Drugs

The following drugs were all obtained from Sigma (St.Louis,Mo.,USA): atropine-sulfat, cadmium chloride-1-hydrate, hexametonium hydrochloride, allopurinol(4-Hydroxypyrazolo[3,4-d]-pyrimidine; HPP), tiron (4,5-Dihydroxy-1,3-Benzene-Disulfonic Acid) disodium salt, α,β -methylene ATP, tetrodotoxin (TTX). Stock solutions of drugs were dissolved in distilled water. Solutions were stored at -20°C . The drugs were diluted in Krebs to the required final concentration on the day of use.

Statistics

Experimental values were expressed as mean \pm SEM. Groups were compared statistically using general linear models of ANOVA followed by post hoc analysis with the Bonferroni test.

Values of $P < 0.05$ were considered to be significant statistically.

Results

EFS evoked contractile responses in rabbit bladder. Mean amplitude of the EFS-evoked contractile responses was 2.23 ± 0.35 g at 8 Hz of stimulation frequency.

Tetrodotoxin (TTX) (3×10^{-6} M), a blocker of Na^{+} channels, abolished the EFS-evoked contractile responses in rabbit bladder strips.

Effects of drugs on neurogenic contractions of the rabbit bladder strips:

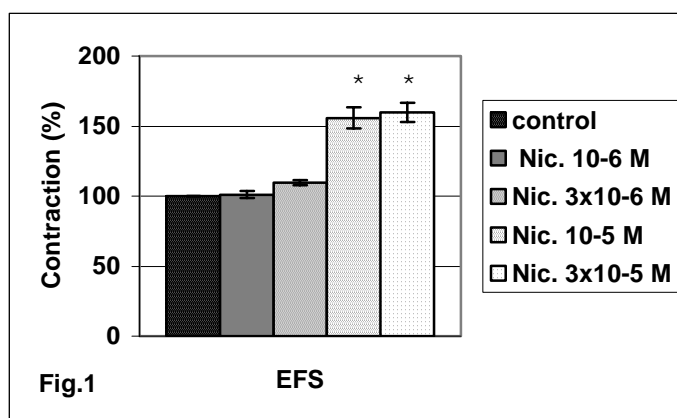
EFS-evoked contractile responses were significantly decreased by atropine (10^{-6} M, 45.91 ± 7.69 %, $p < 0.05$) a muscarinic receptor blocker and α,β -methylene ATP (10^{-5} M, 49.32 ± 1.06 %, $p < 0.05$) a purinergic agonist that desensitise P2X receptors. Atropin and α,β -methylene ATP with together abolished the EFS-evoked responses in rabbit bladder strips.

Hexametonium (10^{-5} M), an unspecific nicotinic acetylcholine receptor blocker, did not alter EFS-evoked responses in rabbit bladder strips.

Effects of nicotine on neurogenic contractions of the rabbit bladder strips:

Nicotine led to increase in the amplitudes of the EFS-evoked contractile responses in a dose dependent manner (3×10^{-6} M, $9,64 \pm 1,93\%$, 10^{-5} M, $55,93 \pm 7,65\%$, 3×10^{-5} M, $59,91 \pm 6,82\%$, $p < 0.05$) and had no effect at the 10^{-6} M concentration (Figure 1). These increase were reproducible and not significantly different after an hour. No tachyphylaxis developed.

Figure 1: Effects of the different concentrations of nicotine (Nic.) [nicotine 10^{-6} (n=10), nicotine 3×10^{-6} (n=10), nicotine 10^{-5} (n=10), nicotine 3×10^{-5} (n=10)] on the EFS-evoked responses. Each point is expressed as a percentage of the control and is given as the mean \pm SEM (*, $p < 0.05$).

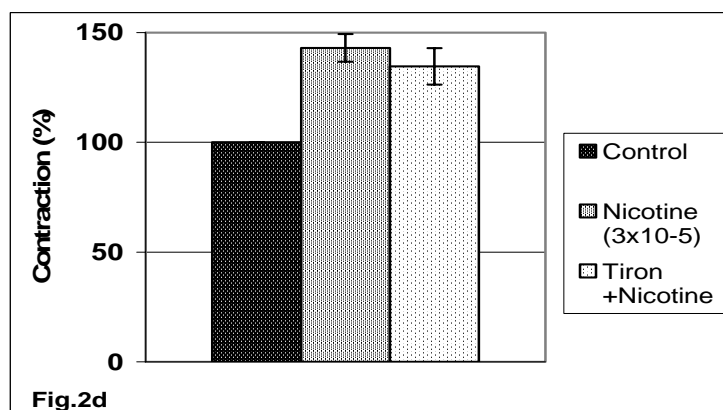
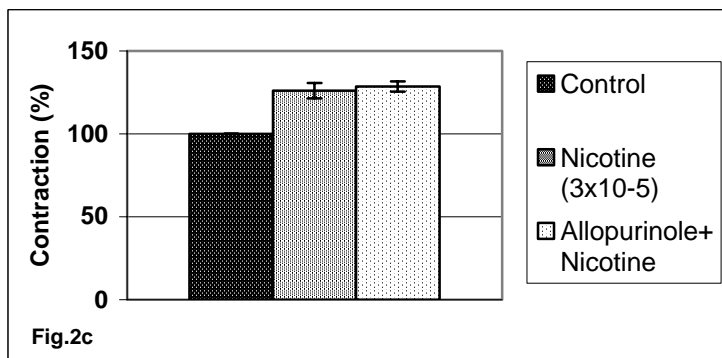
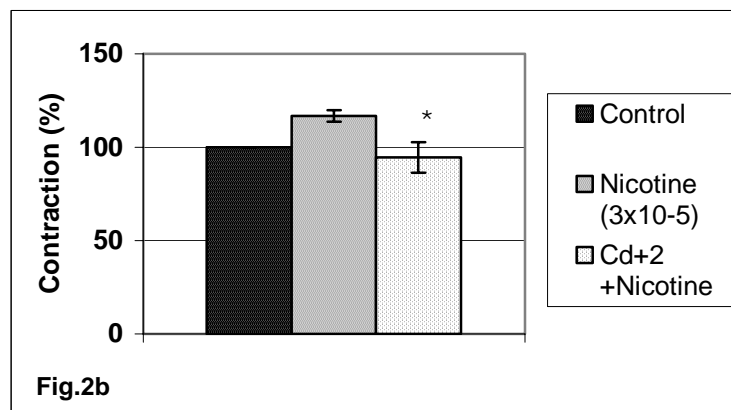
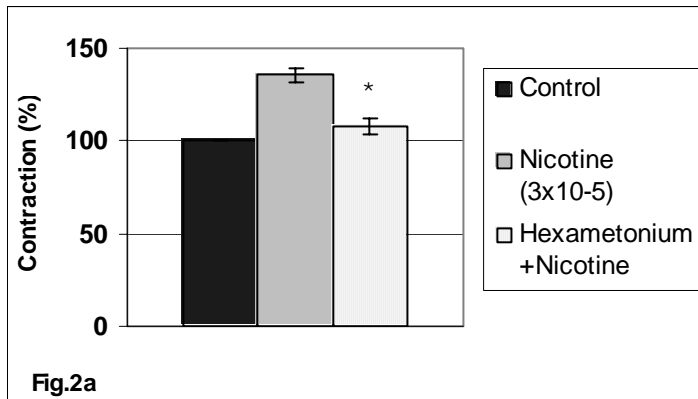


Effects of drugs on nicotine induced EFS-evoked contraction increases:

Hexamethonium (10^{-5} M, $25.59 \pm 11.3\%$, $p < 0.05$) and Cd^{2+} (10^{-4} M, $19.04 \pm 7.48\%$, $p < 0.05$) inhibited nicotine-induced increases in EFS-evoked responses (Figures 3a, 3b) whereas allopurinol (10^{-6} M), axanthine oxidoreductase inhibitor, and tiron (3×10^{-5} M), a selective superoxide quencher, had no effects (Figures 3c, 3d).

Nicotine produced a relaxation at the only high concentrations on the preparations which were precontracted with histamine (Nicotine 10^{-4} M; $16,19 \pm 2,71\%$) but did not produce a change at the concentrations we used. Similarly, nicotine had no contractile effects, *per se*, on quiescent preparations at the concentrations we used. But at the high concentrations nicotine led to contractile responses (10^{-4} M; $0,86 \pm 0,32$ g).

Figure 2: Effects of the different drugs [hexametonium 10^{-5} M (Fig 2a) (n=8), Cd^{+2} 10^{-4} M (Fig.2b) (n=8), allopurinol 10^{-6} M (Fig 2c) (n=8) and tiron 3×10^{-5} M (Fig 2d) (n=8)] on the nicotine (3×10^{-5} M)-induced increase in EFS-evoked contractile responses. Each point is expressed as a percentage of the control and is given as the mean \pm SEM (*, $p < 0.05$).



Discussion

In rabbit bladder parasympathetic postganglionic neurotransmission which is mediated by co-transmit of acetylcholine and ATP, produces contractions. In this co-transmission, ATP activates postganglionic P2x-purinoceptors and acetylcholine activates postganglionic muscarinic receptors (12-15). Pharmacological analysis of field stimulation-induced mechanical responses in isolated smooth muscle strips is a useful *in vitro* technique for clarifying autonomic innervation. In the present experiments, atropine and α - β methylen ATP antagonize EFS-evoked contractile responses, indicating the presence of cholinergic and purinergic activities in rabbit bladder. To evaluate the involvement of cholinergic activation in EFS-evoked contractions, atropine was used to block muscarinic receptors and to evaluate the involvement of purinergic activation in EFS-evoked contractions, α - β methylen ATP was used to block purinergic receptors (P2x). These antagonists inhibited the EFS-induced contractile responses and with together they abolished the EFS-evoked responses in rabbit bladder strips indicating that cholinergic and purinergic co-transmission is prominent in rabbit bladder as it has been shown previously (12-15).

Nicotine which is a nonspecific nAChRs agonist, potentiated the contractile responses elicited by the EFS in a dose dependent manner in our experiments. The potentiation was reversible and in the case of nicotine, no tachyphylaxis developed. The nicotine-induced potentiation was characterized by a rapid onset and an initial, transitory peak. Although nicotine has been shown to have a facilitatory effect on neurotransmitter release through acting on nAChRs, little is known about the exact mechanisms of this action (16,17). Our previous studies, nicotine potentiated the EFS-evoked contractile responses similarly in rabbit gastric fundus and myometrium (unpublished). In the present experiments TTX, a blocker of Na⁺ channels, abolished the EFS-evoked responses. Hexamethonium, a nonspecific nAChRS antagonist, prevented the potentiation caused by nicotine. These results indicating that EFS-evoked responses are induced by nerve stimulation and nAChRs are responsible for the potential effect of nicotine. Previously nAChRs have been reported to be involved in the nicotine-induced enhancement of neurotransmitter release in both central and peripheral neurons (2,18). In rat striatum it was demonstrated that nicotine-evoked dopamine release was prevented by TTX (19).

In the present experiments indicating that the responses. It seems likely that nicotine acts on the neuronal nAChRs located on the parasympathetic and purinergic ganglia, thereby evoking the release of acetylcholine and ATP from parasympathetic and purinergic nerve terminals in the rabbit bladder. Previously it was shown that ganglionic nicotinic receptors are involved in nicotine induced release of neurotransmitters (8,19).

The effect of cadmium (Cd²⁺) which blocks all known voltage-gated calcium channels involved in the EFS-evoked responses, was tested to confirm the involvement of these channels in neuro-effector transmission. Contractions elicited by EFS and nicotine-induced increase in EFS-evoked contractions were reduced by Cd²⁺ at the concentration of 10⁻⁴ M. These results suggest that voltage-gated Ca²⁺ channels (VGCCs) are required for the excitatory neuro-effector transmission in the rabbit bladder and plays a role in the enhancement of EFS-evoked contractions by nicotine. Nicotine triggers the influx of Ca²⁺ through the ligand-gated channel and/or VGCCs via activation of nAChRs. Then the release of Ca²⁺ from intracellular calcium stores is triggered. All of these Ca²⁺ activation plays an important role in the action of nicotine via activation of the presynaptic nAChRs and as a result neurotransmitter release increases (17,20-22). In this study, it is likely that nicotine

enhances EFS-evoked responses by facilitating neurotransmitters release from nerve terminals by a mechanism dependent from the influx of Ca^{2+} via voltage gated Ca^{2+} channels (VGCC).

In the present study we investigate the effects of ROS on nicotine induced peripheral neurotransmitter releases by diminishing ROS generation with using allopurinol , a xanthine oxidoreductase inhibitor, and tiron , a selective superoxide quencher. Both allopurinol and tiron did not alter EFS-evoked responses and nicotine-induced increases on EFS-evoked responses in rabbit bladder strips. These results suggest that ROS have no effects on neuro-effector transmission and enhancement of EFS-evoked contractions by nicotine in rabbit bladder. But in different studies inhibitor effect of ROS on synaptic neurotransmission was demonstrated (9-11). It was shown that hydrogen peroxide inhibits neurotransmitter release possibly by decreasing Ca^{2+} entry via presynaptic Ca^{2+} channels (9). Also it was demonstrated that endogenous Ca^{2+} dependent H_2O_2 production inhibits synaptic dopamine release in guinea pig striatal slices (10). Similarly H_2O_2 inhibited the glutamate release in cerebrocortical synaptosomes from guinea pig (11).

In conclusion, nicotine increased the EFS-evoked contractile responses possibly by facilitating neurotransmitters release from nerve terminals by a mechanism dependent on the influx of Ca^{2+} from voltage gated Ca^{2+} channels (VGCC) via activation of nAChRs in isolated rabbit bladder. ROS do not play a physiological role on the regulation of these neurotransmitter release. nAChRs subtypes involved in nicotine-induce neurotransmitter release and modulation of this mechanism needs further investigation.

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