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# Role of nicotinic acetylcholine receptor subtypes on nicotine's enhancing effect on electrical field stimulation elicited contractile responses in rabbit urine bladder

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**Abstract. – OBJECTIVE: This study aims to investigate the contribution of presynaptic nicotinic acetylcholine receptors (nAChRs) sub-types to nicotine-induced enhancement in electrical field stimulation (EFS) EFS-mediated contractile responses in rabbit urine bladder smooth muscle preparations.**

**MATERIALS AND METHODS: Rabbit urine bladder smooth muscle strips were placed in organ baths containing 20 ml of an aerated Krebs-Henseleit solution, and contractions were recorded using isometric force displacement transducers. Following the acquisition of control EFS (60 V, 8 Hz, 1 ms) responses, nicotine was added to the bath at a $3\times10^{-5}$ M concentration, and EFS responses were obtained. The effect of nAChR antagonists on nicotine-induced augmentation in EFS-mediated responses was investigated in the presence of hexamethonium, dihydro-β-erythroidine, mecamylamine, and α-bungarotoxin.**

**RESULTS: Tetrodotoxin (TTX; $10^{-6}$ M) completely blocked EFS-induced contractile responses in smooth muscle strips. Similarly, Atropine ($10^{-6}$ M), when administered with α,β-methylene adenosine triphosphate (α,β-methylene-ATP) ($10^{-5}$ M), completely blocked EFS responses. Nicotine significantly enhanced EFS-mediated contractile responses (23.67% ± 1.75). Nicotine-induced increases in EFS responses were largely inhibited by hexamethonium, mecamylamine, and dihydro-β-erythroidine, whereas α-bungarotoxin only partly inhibited these enhancements.**

**CONCLUSIONS: These findings demonstrate that EFS-induced neurogenic contractions in rabbit urine bladder smooth muscle strips are mediated by purinergic and cholinergic transmissions, and the α4β2, α3β4, and α7 sub-types of nAChRs contribute to the enhancement effect of nicotine on EFS-induced contractile responses.**

*Key Words:*
Nicotine, Electrical field stimulation, Nicotinic acetylcholine receptors, Urine bladder.

## Introduction

Nicotine, a biologically active substance also found in tobacco, is a non-specific agonist of nicotinic acetylcholine receptors (nAChRs), which are dispersed throughout both the central and the peripheral nervous systems and in non-neuronal tissues, including the immune system, intestinal epithelium, lungs, and skin[1-3]. Ganglionic nAChRs are heterogeneous in their functional and pharmacological characteristics, a finding consistent with the expressions of various nAChR subunits. Seventeen types of nAChR subunits (α1-10, β1-4, γ, δ, and ε) have been identified to date[4,5]. Autonomic ganglia express α3 and β4 at high levels, but they also contain mRNA transcripts for the α4, α5, α7, and β2 nAChR subunits[6-8].

There are numerous reports that define the modulating properties of nAChRs on synaptic neurotransmission in the body[2]. We have previously demonstrated that nicotine increased electrical field stimulation (EFS)-evoked contractile responses, possibly by facilitating neurotransmitter release from nerve terminals in rabbit bladders in a nitric oxide- and prostaglandin-independent manner[9]. We have also shown that nicotine enhances the contractile responses of the rabbit


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gastric fundus, corpus cavernosum, and myometrium in response to EFS[10-12].

Neuronal nAChRs contribute to the control of bladder function by mediating fast synaptic transmissions between preganglionic and postganglionic bladder neurons[13]. Autonomic ganglia are found to express more than one nicotinic receptor subtype[6-8], and bladder ganglia likely express multiple nicotinic receptor subtypes. For instance, mRNA expression of α3, α5, α7, β3 and β4 nicotinic receptor subunits were identified in rat urothelial cells[14].

In the present study, we aimed to characterize the possible involvement of nAChR sub-types in nicotine-mediated EFS-evoked contractile response augmentation in rabbit urine bladders.

## Materials and Methods

### Animals and Tissue Preparation

In total, 12 New Zealand albino male adult rabbits (3.0 ± 0.5 kg body weight) aged between 4 and 6 months were used in the study. They all received standard laboratory food and tap water ad libitum. The experimental protocol for the study was reviewed and approved by the Local Ethics Committee for Animal Experiments of the Gazi University (G.U.ET-05.073). Rabbits were sacrificed by an intravenous overdose of thiopental sodium (50 mg/kg). Their urine bladders were quickly excised, opened lengthwise, and emptied. Four uniform longitudinal detrusor strips (15 mm long × 3 mm wide) were obtained from the bladders after adherent fat and connective tissues were removed.

### Organ Chamber Experiments

All tissues were then placed in 20-ml organ baths containing a Krebs-Henseleit solution (composition in mmol/l: 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; and glucose monohydrate, 11), maintained at 37°C, and aerated with 95% $O_2$ and 5% $CO_2$ during the entire experiment. Each strip was mounted under 1 g of isometric resting tension. Tissues were equilibrated for 60 min, with frequent washes at 15-min intervals. Isometric contractions were evoked by EFS using a pair of platinum electrodes with a stimulation frequency of 8 Hz and 10-s trains of impulses delivered every 2 min. Pulses of 1-ms duration (60 V) were delivered by a stimulator (STPT 03, May Research Stimulator; COMMAT Iletisim Co., Ankara, Turkey). EFS-evoked responses were recorded by isometric force displacement transducers (FDT10-A, May IOBS 99; COMMAT Iletisim Co.) connected to an online computer with a four-channel transducer data acquisition system (MP30B-CE; BIOPAC Systems Inc., Santa Barbara, CA, USA) using a software (BSL PRO v 3.6.7; BIOPAC Systems Inc.) that also had the capacity to analyze the data.

To test the contribution of cholinergic and purinergic components, the tissue was treated either with atropine ($10^{-6}$ M), a muscarinic receptor blocker, or with α,β-methylene-ATP ($10^{-5}$ M), a purinergic agonist that desensitizes purinergic receptors, 30 min after the EFS-induced responses reached a steady state. The effects of tetrodotoxin (TTX) ($10^{-6}$ M) on EFS-induced responses were also tested. Nicotine was then added to the bath media at a concentration of $3\times10^{-5}$ M (n=8). To avoid any possible habituation effect or tachyphylaxis, EFS was terminated after five contractions were achieved and the preparations were washed for 1 h at every 15-min interval. Following the tissue wash, EFS was delivered again and the same experimental procedure was performed with the same tissue in the presence of hexamethonium (a non-specific nAChR antagonist; $10^{-4}$ M, n = 5), dihydro-β-erythroidine (α4β2 nAChR antagonist; $10^{-5}$ M, n = 5), mecamylamine (α3β4 nAChR antagonist; $10^{-5}$ M, n = 5), or α-bungarotoxin (α7 nAChR antagonist; $3 \times 10^{-7}$ M, n = 5). Antagonists were added to the organ baths 30 min prior to nicotine administration.

### Drugs

All drugs (atropine sulfate, α,β-methylene-ATP, TTX, nicotine, hexamethonium hydrochloride, dihydro-β-erythroidine, mecamylamine, and α-bungarotoxin) used in the study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of drugs were dissolved in distilled water and stored at –20°C until use. The drugs were diluted in Krebs solution to the required final concentration on the day of use.

### Statistical Analysis

Nicotine-induced increases were expressed as percentages with regard to control. The maximum of the five EFS-evoked contraction responses after nicotine administration was taken into consideration. Nicotinic ACh receptor antagonist responses were normalized with respect to maximal responses obtained after nicotine em-

ployment. Data were expressed as the mean ± SEM. Groups were compared statistically using general linear models of an analysis of variance (ANOVA), followed by a post-hoc analysis with the Bonferroni test. $p < 0.05$ was considered statistically significant.

## Results

The EFS-induced contractile responses were measured in rabbit urine bladder tissues. The mean magnitude of the EFS-induced contractile responses was 2.08 (±0.17) g at an 8-Hz stimulation frequency. EFS-induced contractile responses were abrogated by $10^{-6}$ M TTX. EFS-induced contractile responses were decreased significantly in response to administration of atropine ($10^{-6}$ M, 53.39 ± 3.33) or α,β-methylene-ATP ($10^{-5}$ M, 45.56% ± 0.67%; $p<0.05$). The combined administration of atropine and α,β-methylene-ATP abrogated the EFS-induced responses in rabbit bladder tissues (Figure 1).

Nicotine ($3\times10^{-5}$ M) increased the amplitudes of EFS-induced contractile responses (23.67% ± 1.75%, $p<0.05$) (Figure 2). These enhancements were reproducible and did not change significantly during the second period of EFS after washing. No tachyphylaxis was observed during the experiments. Nicotine-induced EFS contractile responses were largely inhibited by hexamethonium, mecamylamine, and dihydro-β-erythroidine (percentages of inhibition: 84.18% ± 7.81%, 83.94% ± 9.28%, and 72.43% ± 12.05%, respectively; $p < 0.05$). Further, α-bungarotoxin partly inhibited nicotine-induced responses (percentage of inhibition: 24.83% ± 4.73%; $p<0.05$) (Figure 3). Hexamethonium, dihydro-β-erythroidine, mecamylamine, and α-bungarotoxin had no significant effect on EFS-induced contractile responses.

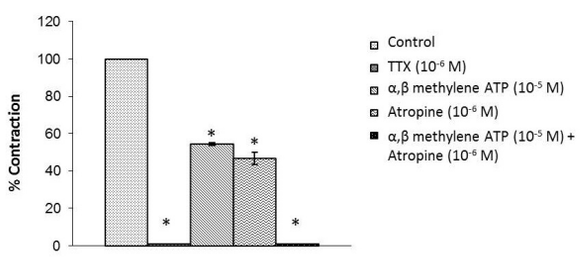


**Figure 1.** The effects of sodium channel blocker TTX, purinergic receptor desensitizer α,β-methylene ATP, muscarinic receptor antagonist Atropine, and the combination of α,β-methylene ATP and Atropine on EFS induced contractile responses in rabbit urine bladder strips. TTX and α,β-methylene ATP - Atropine combination completely blocked EFS induced responses. All points are given as the means ± SEM (*, $p< 0.05$).

## Discussion

Nicotine, a non-specific nAChR agonist, was found to enhance EFS-mediated contractile responses in this study in a reversible manner and without any detectable tachyphylaxis developed. Nicotine was shown to increase EFS-induced responses transiently in various tissues, such as rabbit bladder, gastric fundus, corpus cavernosum, and myometrium[9-12]. TTX, a $Na^+$ channel blocker, completely abrogated the EFS-induced responses in the present experiments, suggesting these responses were induced by nerve stimulation. We have previously demonstrated that TTX is capable of abolishing EFS-mediated responses in the bladder. In that particular study, we also showed that nitric oxide and prostaglandins did not contribute to the nicotine-induced enhancement of EFS responses, and cadmium, a voltage-gated calcium channel blocker, reduced the aforementioned effects of nicotine, suggesting the crucial role of calcium influx through voltage-gated calcium channels[9]. In the present study, nicotine's effect on EFS-mediated contractile responses was strongly diminished by hexamethonium, mecamylamine, dihydro-β-erythroidine (84.18% ± 7.81%, 83.94% ± 9.28%, and 72.43% ± 12.05%, respectively; $p<0.05$), and, to a lesser extent, α-bungarotoxin (24.83% ± 4.73; $p<0.05$).

Parasympathetic transmission in the rabbit bladder is mainly mediated by postganglionic nerves that use (co-transmit) ACh and ATP[15,16]. Thus, EFS-induced mechanical responses in smooth muscle preparations are successfully used for the investigation of autonomic innervation. The present work aimed to evaluate the involvement of cholinergic activation in EFS-mediated contractile responses in the presence of atropine, a muscarinic receptor blocker. Furthermore, for the evaluation of the involvement of the purinergic system in EFS-mediated contractions, α,β-methylene-ATP was used for blocking purinergic receptors. These agents individually inhibited EFS responses and, when administered together, abrogated EFS-induced responses in the rabbit bladder, suggesting that parasympathetic system is a prominent regulatory factor in

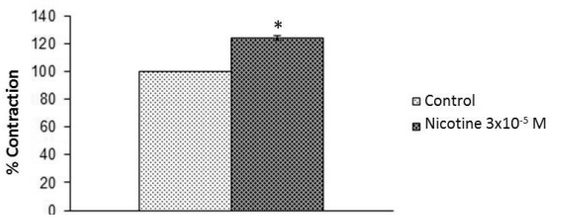


**Figure 2.** Effect of nicotine ($3x10^{-5}$ M) on EFS induced contractile responses. Each point is expressed as a percentage of the control and is given as the mean ± SEM (*, $p< 0.05$).

the rabbit bladder via cholinergic and purinergic co-transmissions. This finding is consistent with previous researches[15,16].

The nAChRs play a crucial role in the regulation of bladder functions. Fast synaptic transmissions in autonomic ganglia and nAChR activation in the parasympathetic neurons of the bladder yield detrusor muscle contractions[17]. Additionally, nAChRs are also found in urothelium[14], dorsal root ganglion sensory neurons[18], and primary afferent terminals of spinal medulla[19], suggesting a possible functional role for nAChRs in the modulation of bladder function, either in the periphery or in the central nervous system

We have previously demonstrated that the α3β4 and α4β2 subunits of nAChRs play a role in the nicotine-induced augmentation of EFS-mediated contractions in rabbit gastric fundus[20]. In another report[21], the α3β4, α4β2, and α7 subunits of nAChRs were shown to contribute to nicotine's augmentation effect on EFS-mediated relaxation responses in rabbit corpus cavernosum. However, there is insufficient information on the subunit composition of nAChRs being expressed in the pelvic plexus and intramural ganglia of the rabbit bladder. Studies carried out in knock-out mice lacking the α3 nAChR subunit alone or the β2 and β4 subunits together suggest α3, β2, and β4 are among the most important nAChRs involved in the regulation of the urinary system. Both α3-/- and β2-/- β4-/- knock-out animals are prone to develop severe bladder distension on post-natal day 2, followed by overflow incontinence. In animals that managed to survive more than a week were urinary contaminations, and their urine was often cloudy; in some cases, there was a massive bladder stone formation that completely occupied the bladder[17,22,23]. Nicotine was found to fail to induce bladder smooth muscle contractions in α3- and β2β4-null mutant mice, whereas EFS or muscarinic agonist administration elicited contractions. Probable functional ACh receptors composed of the α3, β2, and β4 subunits include the α3β2 and α3β4 nicotinic receptor sub-types, also known as α3* receptors[22,23]. Beckel et al[14] claim that α3* nicotinic receptors in the bladder epithelium release excitatory mediators of the micturition reflex; besides, the intravesical administration of hexamethonium (ganglionic α3* nicotinic receptor antagonist) can increase inter-contractions.

The expression levels of nicotinic subunits in the bladder were analyzed in several studies[14,24,25]. Beckel et al[14] showed the mRNA expression of the α3, α5, α7, β3, and β4 nicotinic subunits in rat urothelial cells using RT-PCR. In addition, the protein expression levels of the α3 and α7 subunits were shown in rat urothelial cells by western blotting. In another study, the expressions of each subunit (α, β, and ε) were demonstrated in rat whole bladder using real-time PCR[24]. Bschleipfer et al[25] also showed the expression and distribution of all MR sub-types (M1R-M5R) and the nAChR α7, 9, and 10 subunits in the human urothelium at both the mRNA and protein levels.

Masuda et al[26] showed *in vivo* that nicotine employed via intra-vesical route had a decreasing effect on inter-contraction intervals, and this effect was dose-dependent. Further, the co-application of mecamylamine and pretreatment with capsaicin eliminated the mentioned effects. This latter paper suggested an induction of detrusor muscle activity via nicotinic receptor activation over capsaicin-sensitive C-fiber afferents of the bladder. The observed up-regulation of the α3, β2, and β4 subunits of nicotinic receptors in rat urine bladders in response to multiple administrations of nicotine may highlight the possible involvement of tobacco smoking in the development of lower urinary tract symptoms[26-28].

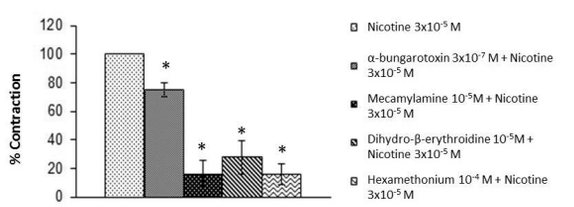


**Figure 3.** Effects of nicotinic receptor antagonists on nicotine induced increases in EFS-elicited contractile responses in bladder stips. Data is presented as (%) change in respect with nicotine induced maximal responses. All data points are given as the means ± S.E.M. (*, $p< 0.05$).

## Conclusions

In this study, nicotine increased EFS-induced contractile responses in the isolated rabbit urine bladders. Hexamethonium, mecamylamine, and dihydro-β-erythroidine largely inhibited the nicotine-mediated enhancement of EFS-induced contractile responses, while α-bungarotoxin partly inhibited nicotine's mentioned effect. These findings demonstrate that EFS-induced neurogenic contractions in rabbit urine bladder smooth muscle strips are mediated by purinergic and cholinergic transmissions, and the α4β2, α3β4, and α7 sub-types of nAChRs contribute to the enhancement effect of nicotine on EFS-induced contractile responses. Further investigation are needed to demonstrate the existence of the α3β4, α4β2, and α7 sub-types of nicotinic acetylcholine receptor proteins, which play a role in cholinergic/purinergic neurotransmission in the bladder.

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### Conflict of Interest

The Authors declare that they have no conflict of interests.

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