



RESEARCH ARTICLE

**INVESTIGATION OF THE ACTION MECHANISM OF AMMONIUM PYRROLIDINE
DITHIOCARBAMATE ON RAT TRACHEA SMOOTH MUSCLE CONTRACTION-
RELAXATION RESPONSE**

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ABSTRACT

Ammonium pyrrolidine dithiocarbamate (APDTC) is a suppressive thiol compound of Nuclear Factor kappa B (NF-κB) and works to prevent infections, regulate oxidation, prevent cell death, affects on viruses. Therefore, it is recommended to avoid harmful practices and to give importance to active substance research in medical ethics. In postoperative care, respiratory system relaxation treatments are important. There is insufficient research into how the functions of APDTC are regulated in human and animals. In our study, we aimed to evaluate the function of APDTC in rat trachea smooth muscle. 70 male Wistar albino rats were used. The rats were euthanized by giving anesthesia and after applying cervical dislocation the trachea was separated, connective tissues of trachea were separated and placed in Krebs solution. Potassium chloride (KCl), Acetylcholine (ACh), APDTC, Atropine, Phentolamine, Propranolol, Nifedipine and Tetraethylammonium (TEA) were added in to the solution. Eventually, APDTC produced a relaxation response in the tracheal smooth muscle induced by ACh, but this relaxation was not statistically significant. In our study, Half maximal effective concentration (EC50) dose was found to be ineffective. APDTC did not induce L-type Ca²⁺ channel and K⁺ channel receptors to differentiate relaxation response by producing a cholinergic-adrenergic effect. We think that APDTC did not affect these channels. Further studies with the use of different doses are recommended.

Keywords: APDTC, trachea, adrenergic, cholinergic, Ca²⁺ channels, K⁺ channels.

1. INTRODUCTION

Asthma is one of the most common and most often encountered diseases and generally affecting young people in developed and developing countries. Smoking, air pollution, gender, socioeconomic status, exposure to dust, smoke and gases due to occupational encountering are the factors that cause

asthma [1]. According to the Turkish Thoracic Society-Asthma and Treatment Guidelines, asthma is a disease characterized by shortness of breath, chest tightness, coughing and bronchial overactivity in the form of seizures with chronic airway inflammation and is a high cost disease [2]. It is included in the category of serious diseases that results in death if untreated [3]. Bronchodilator agents such as antagonists, methylxanthine and corticosteroid drugs are used during the treatment of asthma. Bronchodilator agents provide relaxation of bronchial smooth muscles [3]. The effect of TEA and K^+ on contractions with histamine and ACh in porcine isolated trachea were compared [4]. While neither TEA nor K^+ increased continuous depolarization with histamine (or induced by acetylcholine), released form of depolarizations were often observed in the presence of TEA. Verapamil and Ca^{2+} Krebs solution reduced histamine contractions and reduced or eliminated the effect of TEA and K^+ on histamine-induced contractions. These results suggest that different contraction mechanisms are masked by histamine and acetylcholine. It appears to be linked to mechanisms that are histamine-mediated, sensitive to TEA and high K^+ and possibly including increased Ca^{2+} translocation along the plasma membrane [4]. In vitro study in rabbit trachea smooth muscle determined that the relaxant effects of β -adrenergic receptor agonists as isoprenaline and salbutamol were caused by the activation of K^+ channels and the relaxant effects of phosphodiesterase inhibitors as theophylline and aminophylline by K^+ channels were less effective [5]. Smooth muscles are found in the structural architecture of organs such as uterus, blood vessels, intestines, trachea, bladder, stomach, penis and clitoral cavernosal sinuses. They receive neural innervation from the autonomic nervous system. Hormonal and paracrine agents, autocrine and local chemical agents further control their contraction [6]. Smooth muscle is composed of cells with a diameter of 1-5 micrometers and only in 20-500 micrometers length [7]. Smooth muscles are composed of long fibers in some organs and short fibers in others [8]. The nucleus is large and at the middle in the smooth muscle. During the embryological development of smooth muscle, precursor cells are not fused and they develop separately in a single nucleus center [9]. Muscle fibers can be in longitudinal, circular and oblique forms [10]. They contain actin and myosin filaments as well as intermediate filaments such as desmin, vimentin and filamin. The adjacent smooth muscle cells are connected by gap junctions. The sarcoplasmic reticulum is the site where Ca is stored in the smooth muscle cell. The smooth muscle cell has both T and L channels [3]. Smooth muscles contain thrombomyosin but lacks troponin. The numbers of mitochondria are low, so glycolysis is used most of the time [11].

The trachea is the organ located after the larynx that transmits air to the bronchi [12]. Asthma is a chronic inflammatory disease of the airways, where mast cells, eosinophils and T lymphocytes and many other cells playing a role in the disease [13]. A neural modulator system composed of various neuropeptides released as cotransmitters from the autonomic nervous system is called NANC System. i-NANC system in human airways is the only neural expansion pathway that relaxes the airway smooth muscle because there is no functional sympathetic innervation. Adrenergic antagonists can not interfere with this system [14]. Excitatory Nonadrenergic-Noncholinergic System (e-NANC) in some species produces bronchoconstrictor effects that cannot be inhibited by atropine, but it can be inhibited by the breakdown of emotional neuropeptides through capsaicin and tachykinin antagonists. The response is produced via secretion of tachykinines released from the sensory nerves of the airway [14].

Nitric oxide (NO) acts as an expander of the airways and is the neurotransmitter of the expanding nerves in the human airways. On the other hand, NO increase in plasma exudation may cause harmful effects on the respiratory tract by vasodilation and also cause inflammatory responses during asthma [15]. NO is a highly lipophilic molecule that can easily pass through the membranes. Nitric oxide sensor-I (NOS-I) is found in neural tissues and Nitric oxide sensor-III (NOS-III) is found in vascular

endothelium. Nitric oxide sensor-II (NOS-II) or Inhibitory Nitric oxide sensor (i-NOS) is found in the airway epithelium and various other cells and is not Ca-dependent. Once synthesized, NO rapidly migrates to target tissues and activates the guanylate cyclase enzyme in the cells and increases the “cyclic guanosine monophosphate” ratio, which provides smooth muscle contraction, by acting as a neurotransmitter of i-NANC neurons with the resulting effect within the smooth muscle cell. There are three neural mechanisms of the airways that cause bronchoconstriction as cholinergic (acetyl choline), α -adrenergic (norepinephrine) and e-NANC (neurokinin) systems. On the other hand, β adrenergic (epinephrine) and iNANC as vasoactive intestinal peptide (VIP) and NO are the mechanisms that cause bronchodilatation. The i-NANC neural system is actively present intermittently in the proximal airways, and this is the bronchodilator system found in human airways. NO in asthma is predominantly associated with lower respiratory tract and increased NOS-II activation. NO rates may be useful in differentiating asthma from other causes of chronic coughing [16]. Transient receptor potential (TRP) channel subtypes are TRP cononical (TRPC) with seven different sub-channel groups, TRP vanilloid (TRPV) with six different sub-channels, TRP polycystein (TRPP) with three different sub-channels, TRP mucolipin (TRPML) with three different sub-channels, TRP ankyrin (TRPA) with one different sub-channel and TRP melastatin (TRPM) with eight different sub-channel groups. The majority of these ion channels are non-selective ion channels that are simultaneously permeable to Na^+ and Ca^{+2} . In addition, there are twenty-eight types of TRP channels in mammals and they can be grouped in six sub-families [17, 18].

Atropine is a competitive antagonist of muscarinic (M) receptors of ACh in the parasympathetic system. Atropine receptors have five subtypes called M1, M2, M3, M4 and M5 and they show postsynaptic localization in various effector organs and ganglia, or located at the ends of cholinergic and adrenergic nerves [19]. The Ca^{2+} channels affect the passage of calcium ions into the cytoplasm through specific pores opened in response to depolarization of the cell membrane. The introduction of calcium into the cell produces a depolarizing effect, and the accumulation of calcium in cytoplasm increases the secretion of hormones and neurotransmitters and is a chemical activator for muscle contraction and various other calcium-sensitive interactions [20]. Nifedipine is a selective relaxant [21]. Adrenergic receptors are divided into two groups as alpha adrenergic receptors and beta adrenergic receptors. Alpha adrenergic receptors are divided into two groups as alpha 1 and alpha 2. Beta adrenergic receptors are divided into three groups as beta1, beta 2 and beta 3 receptors [22]. Phentolamine is a alpha-adrenergic blocker directing smooth muscle relaxation and it is a imidozine derivative with cholinometric, histaminic and sympathomic activity. Phentolamine and other alpha-adrenergic receptors show competitive effects and provide mild intrinsic activity [23]. Phentolamine has an equal affinity for alpha 1 and alpha 2 receptors. Phentolamine has agonistic activity on musacarinic, histamine H1 and H2 receptors. Phentolamine has a relaxing effect on vascular smooth muscles [24]. Propranolol inhibits all beta adrenergic effects, but does not block alpha adrenergic receptors [24]. Many potassium channels of different molecular structure help regulate potassium conductivity in smooth muscle cells [25]. The potassium channels are blocked by Tetraethyl ammonium, which is supplied externally. Sensitivity to tetraethyl ammonium is different in different types of potassium channels. Tetraethyl ammonium inhibits the return of voltage-dependent potassium channel inhibitor repolarization [26]. Research on how APDTC functions are regulated in humans and animals is insufficient. Therefore, the aim of this study was to investigate the mechanism of action of APDTC in the rat smooth muscle of the trachea by using some agonists and blocker chemical substances with a good designed experimental procedure.

2. MATERIALS AND METHODS

70 male Saki Yenilli Wistar albino (200-250 g) male rats (6-7 months old) were obtained from The Experimental Animals Production Center. The study was approved by Dumlupınar University Animal Experiments Local Ethics Committee (HAYDEK) Decision No. 2015.12.04. Wistar albino male rats allocated from 6-7 months old Saki Yenilli Experimental Animal Production Facility weighing 200-250 grams were grouped according to the chemicals used and placed in cages.

All the experimental groups and procedures are presented first as organ bath preparation and experimental procedures: Male Wistar albino rats were weighed sequentially, euthanized by cervical dislocation, and the neck was opened from larynx to bifurcation, and the trachea was isolated without any damage. The trachea was sampled and placed in a Petri dish with cold Krebs Henseleit physiological solution. Physiological solution of Krebs Henseleit was weighed and then the other chemicals such as $MgSO_4 \cdot 7H_2O$ (0.28 g), KH_2PO_4 (0.32 g), KCl (0.72 g), NaCl (13.8 g), Glucose (10 g), $NaHCO_3$ (4.2 g), $CaCl_2 \cdot 2H_2O$ (0.56 g) were weighed and they were dissolved in other beakers with the help of some water. Then the $CaCl_2 \cdot 2H_2O$ solution was added slowly to the solution containing the other chemicals and was added up to 2 liters. After cleavage of the connective tissues around the trachea, the trachea was isolated in the shape of ring. Organ weight was determined by precision scale. For the trachea placed in the isolated organ bath, the temperature of the organ bath was stabilized at 37 °C. 95% O_2 -5% CO_2 gas mixture was introduced into the isolated organ bath and the viability of the organ was maintained by providing oxygenation. The same conditions were applied to each group. One end of the trachea was placed in the organ bath and the other end to the transducer and 1 gram of tension was applied to it and the responses were recorded with the concentrator recorder device. APDTC was weighed as 0.016429 grams. It was dissolved with water, made up to 10 ml and a 10^{-2} M solution was obtained. 1 ml of this solution was taken and completed to 10 ml and 10^{-3} M stock solution was prepared, and then the stock solution was diluted to 1.5×10^{-6} . 0.031781 grams of phentolamine were weighed. It was dissolved with water, made up to 10 ml and a 10^{-2} M stock solution was prepared. The stock solution was then diluted to 10^{-3} . 0.02958 grams of propranolol was weighed. It was dissolved in some ethanol, made up to 10 ml and 10^{-2} M stock solution was prepared and this stock solution was diluted to 10^{-4} . 0.028938 grams of atropine was weighed. It was dissolved in some ethanol, made up to 10 ml and 10^{-2} M stock solution was prepared and this stock solution was diluted to 10^{-4} . 0.16571 grams of tetraethyl ammonium was weighed. It was dissolved in water, made up to 10 ml and 10^{-1} M solution was obtained. 0.034634 grams of nifedipine were weighed. It was dissolved in water, made up to 10 ml and 10^{-2} M stock solution was prepared and then the stock solution was diluted to 10^{-4} . 0.018166 grams of ACh was weighed. It was dissolved in water, 10^{-2} M stock solution was prepared after completed to 10 ml and stock solution was diluted to 10^{-4} . 0.4473 grams of KCl was weighed. It was dissolved in water, made up to 10 ml and a 6×10^{-1} M solution was obtained.

Experimental protocol for rat trachea smooth muscle contraction and relaxation response measurements are presented afterward as follow: 70 Wistar albino male rats were used. The rats were divided into seven groups of containing 10 animals each, respectively. Separate protocols were applied to the rats divided into groups. It was equilibrated using a krebs solution at every 15 minutes for 60 minutes. The viability of the trachea was controlled by means of KCl solution. It was precontracted using acetylcholine. The groups were as follow: Group 1 was used as the control group and 10 animals were used. Contraction and relaxation were examined. 1.5×10^{-6} M APDTC was used (10 animals). In Group 2, cholinergic receptors was inhibited by cholinergic receptor antagonist

atropine (10^{-6} M). Trachea responses were observed (10 animals). In Group 3, 10^{-5} M alpha adrenoceptors were inhibited by alpha adrenoceptor antagonist phentolamine and trachea response was examined (10 animals). The 10^{-6} M propranolol, the beta adreno-receptor antagonist, was given to the 4th group to prevent the effect of beta-adreno-receptors. Trachea responses were observed (10 animals). In Group 5, 10^{-6} M potassium channel blocker nifedipine blocked the potassium channel. Trachea responses were observed (10 animals). In the Group 6, L-type Calcium channels were blocked with 10^{-3} M TEA, an L-type calcium channel blocker (10 animals). In Group 7, the 10^{-5} M non-selective alpha adrenoceptor antagonist phentolamine, 10^{-6} M beta adrenoceptor antagonist propranolol and 10^{-6} M cholinergic receptor antagonist atropine were administered together for adrenergic receptor and cholinergic receptor blockage. The responses of the trachea were observed (10 animals).

In our experiment, after the preparation stage of our trachea smooth muscle strips, we performed an isolated organ bath application to maintain vitality after one gram stretching process. It was left in a Krebs solution for fifteen minutes. The smooth muscle strip was then allowed to equilibrate for forty-five minutes. Afterward, the vitality of tracheal smooth muscle strips were evaluated with 6×10^{-2} M KCl. After waiting five minutes, the trachea smooth muscle strip was cleaned in the organ bath system to get rid off the effect of KCl by repeating three times. By waiting for ten minutes, 10^{-6} M ACh was applied to the krebs solution to maintain anterior muscle tension. Waiting for ten minutes, 1.5×10^{-6} M APDTC to the control group, 10^{-6} M atropine to the second group, 10^{-5} M phentolamine to the third group, 10^{-6} M propranolol to the fourth group, 10^{-6} M nifedipine to the fifth group, 10^{-3} M TEA to the sixth group and 10^{-5} M phentolamine+ 10^{-6} M propranolol+ 10^{-6} M atropine to the seventh group were applied. Fifteen minutes later, 1.5×10^{-6} M pyrrolidine dithiocarbamate [3] was administered to the other groups except the control group. The obtained data were expressed as mean±standard error and evaluated by applying Kruskal Wallis and Mann-Whitney U tests and then $p < 0.05$ values were considered statistically significant and effective and $p > 0.05$ values were considered statistically not significant and ineffective.

3. RESULTS

In this applied organ bath system research, seven groups were included as follows: control, atropine, phentolamine, propranolol, nifedipine, TEA, and fentolamine+propranolol+atropine (mix) groups (Table 1 and Figure 1). We evaluated the effects of APDTC in the presence and absence of an antagonist or blocker on the trachea smooth muscle strip with the application of 10^{-4} M Acetylcholine. Every antagonist and blocker interaction with each other were tested in our designed experiment.

Table 1. Evaluation of differences between experimental groups of APDTC.

Groups	Control	Atropine	Phentolamine	Propranolol	Nifedipine	TEA	Mix
Control	-	0.762	0.650	0.940	0.762	0.597	0.070
Atropine	0.762	-	0.496	0.762	0.496	0.545	0.034
Phentolamine	0.650	0.496	-	0.450	0.821	0.762	0.199
Propranolol	0.940	0.762	0.450	-	0.473	0.597	0.023
Nifedipine	0.762	0.496	0.821	0.473	-	0.880	0.162
TEA	0.597	0.545	0.762	0.597	0.880	-	0.082
Mix	0.070	0.034	0.199	0.023	0.162	0.082	-

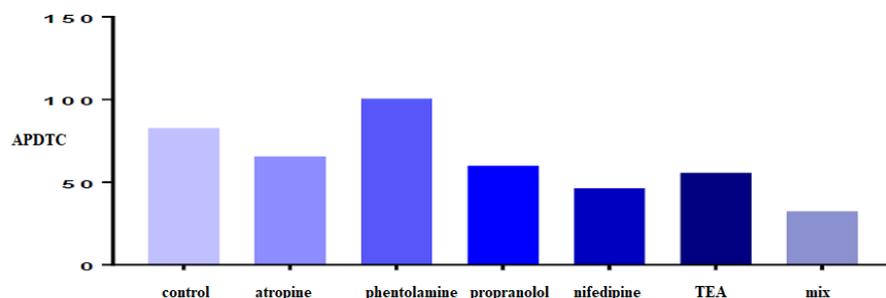


Figure 1. Evaluation of the effects of APDTC in the presence and absence of an antagonist or blocker on the trachea smooth muscle strip with the application of 10^{-4} M Acetylcholine.

There was a statistically significant difference on the tracheal smooth muscle strip between ACh applied precontraction responses and atropine-treated APDTC responses ($p < 0.05$). The relaxation response of APDTC with atropine was observed on the contraction of the tracheal muscle with acetylcholine. However, no statistically significant difference was found between the control group and atropine group ($p > 0.05$). It is revealed that APDTC relaxation responses on the contracted tracheal smooth muscle strip with ACh were not affected by atropine ($p > 0.05$). There was a statistically significant difference between the atropine and propranolol group and the 10^{-6} M dose atropine applied group on the contractions treated with ACh mix group ($p < 0.05$). No statistically significant difference was observed with atropine on contractions induced by ACh in the 10^{-6} M propranolol dose applied group ($p > 0.05$) (Table 2 and Figure 2).

Table 2. Evaluation of the effect of APDTC on trachea smooth muscle by atropine treatment.

Atropine	ACh	Antagonist	APDTC
ACh	-	0.015	0.015
Antagonist	0.015	-	0.070
APDTC	0.015	0.070	-

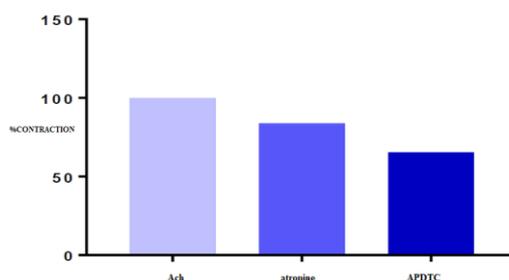


Figure 2. Evaluation of the effect of APDTC in the presence of atropine and atropine on the contraction of the tracheal smooth muscle strip treated with acetylcholine.

There was a statistically significant difference between APDTC responses and ACh precontraction responses with phentolamine treatment in trachea smooth muscle ($p < 0.05$). The contraction response of APDTC were formed in the tracheal smooth muscle strip contracted with ACh and then phentolamine treatment. However, no statistically significant difference was found between the control group and the group under phentolamine intervention. APDTC to the relaxation response to ACh contracted trachea smooth muscle strip with phentolamine application had no effect ($p > 0.05$) (Table 3 and Figure 3).

Table 3. Evaluation of the comparison with the phentolamine only/phentolamine with APDTC administration.

Phentolamine	ACh	Antagonist	APDTC
ACh	-	0.015	0.015
Antagonist	0.015	-	0.406
APDTC	0.015	0.406	-

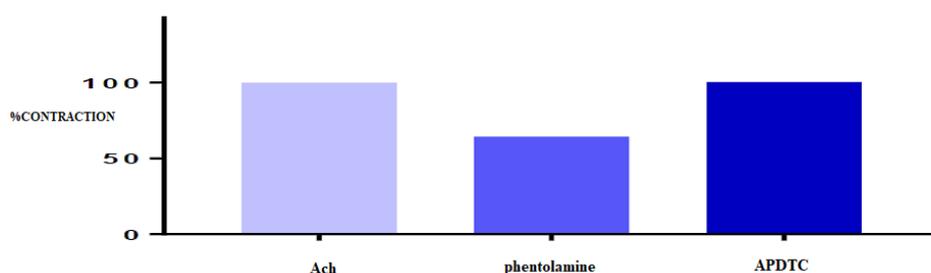


Figure 3. Evaluation of the effect of APDTC in the presence of phentolamine and phentolamine only on tracheal smooth muscle contracted with acetylcholine.

There was a statistically significant difference between APDTC and propranolol administration responses with precontractive responses on trachea smooth muscle treated with ACh ($p < 0.05$). When propranolol was applied to the contraction formed on the tracheal smooth muscle strip with acetylcholine, the relaxation response with APDTC was observed. However, no statistically significant difference was found between the control group and the group under propranolol intervention. Propranolol did not affect the relaxation response of APDTC to Acetylcholine-contracted smooth muscle strip ($p > 0.05$) (Table 4 and Figure 4).

Table 4. Evaluation of the comparison of Propranolol only/Propranolol administration with APDTC.

Propranolol	ACh	Antagonist	APDTC
ACh	-	0.001	0.004
Antagonist	0.001	-	0.705
APDTC	0.004	0.705	-

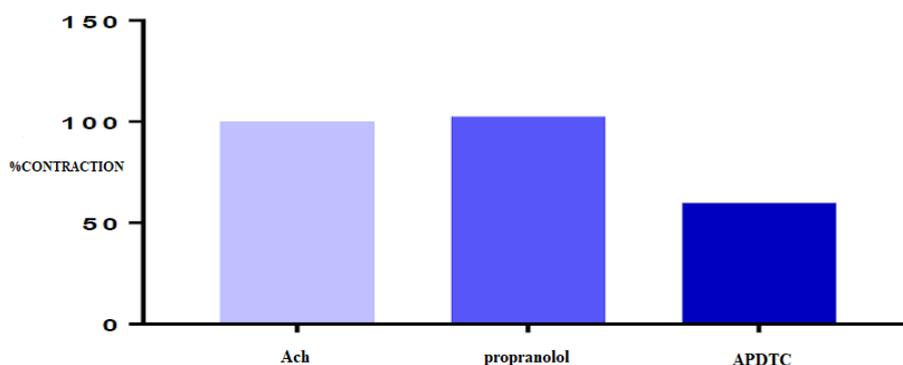


Figure 4. Evaluation of the effect of APDTC by applying propranolol only/propranolol on the contraction of tracheal smooth muscle strip where ACh is applied.

A statistically significant difference was found between the responses of APDTC by the application of nifedipine with the precontraction responses generated with ACh on the tracheal smooth muscle strip ($p < 0.05$). Nifedipine applied to the contractions created in tracheal tissue with ACh has created a contraction response of APDTC. However, no statistically significant difference was found between the control group and the group under nifedipine intervention. Nifedipine did not affect the relaxation responses of acetylcholine-contracted tracheal smooth muscle strip of APDTC ($p > 0.05$) (Table 5 and Figure 5).

Table 5. Evaluation of Nifedipine only/Nifedipine treated APDTC comparison.

Nifedipine	ACh	Antagonist	APDTC
ACh	-	0.001	0.001
Antagonist	0.001	-	0.940
APDTC	0.001	0.940	-

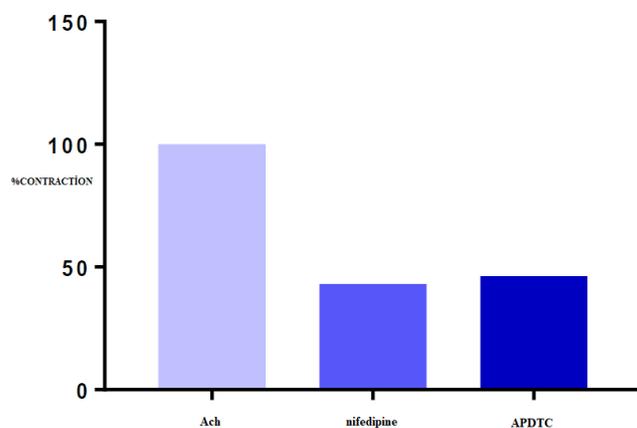


Figure 5. Evaluation of APDTC effect on tracheal smooth muscle strip with nifedipine treatment.

A statistically significant difference was found between precontraction responses with ACh treated tracheal smooth muscle strip and APDTC responses under TEA intervention ($p < 0.05$). APDTC contraction response has occurred with the application of TEA on the contraction created in the trachea muscle strip with acetylcholine. However, there was no statistically significant difference between the control group and the group under TEA intervention. APDTC did not change the relaxation responses of the tracheal smooth muscle strip that was contracted with ACh by TEA administration ($p > 0.05$) (Table 6 and Figure.6).

Table 6. Evaluation of APDTC in TEA only/with TEA application.

TEA	ACh	Antagonist	APDTC
ACh	-	0.001	0.015
Antagonist	0.001	-	0.151
APDTC	0.015	0.151	-

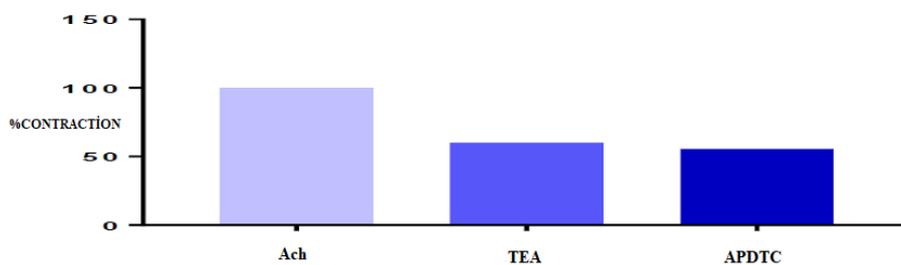


Figure 6. Assessment of the effect of APDTC with treatment of TEA only/TEA on trachea smooth muscle strip contracted with acetylcholine.

A statistically significant difference was found between responses formed by contracting tracheal smooth muscle strip with ACh and responses to APDTC with administration of phentolamine+propranolol+atropine ($p < 0.05$). APDTC relaxation response has occurred by applying phentolamine+propranolol+atropine on the trachea smooth muscle strip contraction treated with acetylcholine. However, the relaxation occurred statistically between the contraction response caused by phentolamine+propranolol+atropine and with the application of APDTC ($p < 0.05$). APDTC did not change relaxation responses on the tracheal smooth muscle strip contracted with ACh with the phentolamine+propranolol+atropine ($p > 0.05$) (Table 7 and Figure 7).

Table 7. Evaluation of comparison of APDTC when Mix only/Mix is applied.

Mix	ACh	Antagonist	APDTC
ACh	-	0.419	0.001
Antagonist	0.419	-	0.004
APDTC	0.001	0.004	-

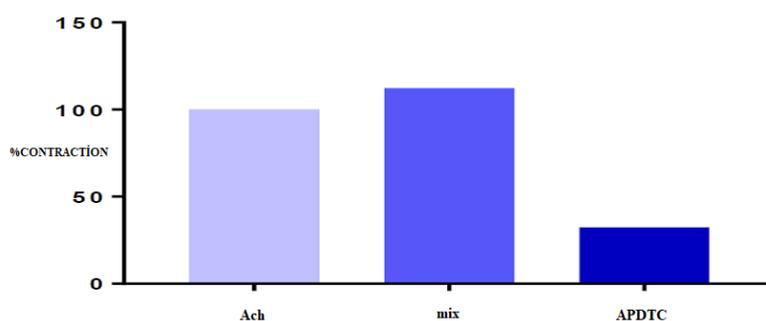


Figure 7. Assessment of the effect of APDTC by applying mix only and with mix on tracheal smooth muscle strip contraction with acetylcholine.

4. DISCUSSION AND CONCLUSION

In this study, the contraction was created by ACh treatment on the trachea smooth muscle strip and then KCl, acetylcholine, APDTC, atropine, phentolamine, propranolol, nifedipine and TEA interventions were made. The effecting function of APDTC on the tracheal smooth muscle strip was evaluated with organ bath application since the evidence-based APDTC mechanism for the relaxation of the trachea smooth muscle strip was not fully examined within previous studies. In this study, the effecting functions of APDTC in 7 designed different groups (control, atropine, phentolamine, propranolol, nifedipine, TEA and phentolamine+propranolol+atropine) were evaluated to find out whether some channels and receptors were used or not on the rat tracheal smooth muscle strip with ACh application. A study investigated the roles of NF- κ B in lung injury initiated by single lung ventilation by treating rats with NF- κ B-specific inhibitor PDTC, and then the NF- κ B pathway was activated in the process after lung injury and the suppression of the NF- κ B pathway prevented the development of lung damage from different routes [27]. It also affected the respiratory functions of APDTC [27]. A research reported that a decrease in smooth muscle contractions in colitis rat colon

stimulated with trinitrobenzene sulfonic acid (TNBS) can be attributed to the decreased activity of the L type Ca^{+2} channel. In this context, they concluded that L-type Ca^{+2} channel dysfunction can be mediated by NF- κ B related pathways [28]. We discovered that 1.5×10^{-6} M APDTC produced a relaxation response in the tracheal smooth muscle.

There is a statistically significant difference between the precontraction responses with ACh and the responses of APDTC with atropine treatment in tracheal smooth muscle. The relaxation response to APDTC with the application of atropine on the contraction created with ACh in the tracheal smooth muscle strip. However, there was no statistically significant difference between the control group and the group that received atropine. APDTC replaced the relaxation responses in the tracheal smooth muscle strip contracted with ACh with a nonselective muscarinic receptor antagonist atropine [29]. Atropine can be considered as an ACh competing antagonist, mostly at the cholinergic nerve endings [29]. APDTC with the dose of 1.5×10^{-6} M was found that it uses muscarinic-cholinergic pathways in the tracheal smooth muscle strip. In our study, a statistically significant difference was found between the precontraction response formed with ACh in the trachea smooth muscle strip and the responses of APDTC when phentolamine was administered. The contraction response of APDTC was shown by the intervention of phentolamine on the contraction created in the trachea smooth muscle strip with acetylcholine. However, no statistically significant difference was found between the control group and the group in the presence of phentolamine. Phentolamine has changed the reflexion responses of tracheal smooth muscle strip contracted with ACh by APDTC. With the intervention of phentolamine, the relaxation response of APDTC has occurred.

If glibenclamide or phentolamine try to block the K^{+} channels opened by cromakalim, such channels are not the same as those having trachealis plasmalemma with their strong rectifying behavior [30]. It has been concluded that each of the glibenclamide and phentolamine provides a selective contrast to the relaxant effect of cromakalim in the Guinea pig trachealis [30]. The effect of fentolamine is not related to the blockade of the $\alpha 1$ or $\alpha 2$ adrenoceptors. However, they stated that phentolamine provided APDTC to cause relief of relaxation. In this context, they stated that APDTC can use some of the α -adrenergic receptors [30]. In our study, a statistically significant difference was found between the precontraction responses with ACh in the trachea smooth muscle strip and the responses of APDTC in the intervention of propranolol. APDTC's relaxation response in the intervention of propranolol on the contraction created with ACh in the tracheal smooth muscle strip. However, no statistically significant difference was found between the control group and the propranolol group. Propranolol did not affect the relaxation responses of APDTC in the tracheal smooth muscle strip contracted with acetylcholine. The α -adrenergic blocker Propranolol is a non-selective beta adrenergic receptor blocker. It blocks competitively with both $\beta 1$ and $\beta 2$ receptors [24]. However, the blocking of β -adrenergic receptors with APDTC by propranolol did not increase the relaxation effect of APDTC. As a result, we can say that APDTC has its effect through these receptors. Propranolol did not affect relaxation response of APDTC's tracheal smooth muscle strip contracted with acetylcholine. There is a significant difference between the APDTC responses in the presence of nifedipine and pre-contraction responses created by ACh in the tracheal smooth muscle. APDTC showed a contraction response in the presence of nifedipine on contraction created in tracheal tissue with ACh. However, there is no significant difference between the control group and the nifedipine administered group. The nifedipine did not alter the APDTC tracheal smooth muscle relaxation responses contracted with ACh. It is understood from other studies that nifedipine directly inhibits smooth muscle contraction with the competitive inhibition of the transmembrane Ca^{+2} flow, thereby preventing the process in which pharmacological (or electrical) stimulation is converted into mechanical contraction. The failure of nifedipine to completely eliminate all restrictions in our experiments may result from the

secretion of histamine-induced Ca^{+2} stores used nifedipine or unaffected by calcium channel blocker, and nifedipine inhibits histamine-induced bronchoconstriction [31]. Nifedipine increased the APDTC effect even more, and blocking the channels could not change this effect. L-type calcium channels do not play a role in the mechanism of action of APDTC. There is a significant difference between the precontraction responses generated by ACh in the tracheal smooth muscle and the responses of APDTC in the presence of TEA. In the presence of TEA on contraction in tracheal tissue with ACh, APDTC showed a contraction response. However, there is no significant difference between the control group and the TEA administered group. TEA did not change APDTC's tracheal smooth muscle relaxation responses, which were contracted with ACh. It can be thought that 1.5×10^{-6} M dose of APDTC uses different routes other than potassium channels.

Cromakalim, an ATP-dependent K^{+} channel opener, investigated for contraction mechanisms originating from Butylidenephthalide (Bdph), significantly increased basal tension due to Bdph [32]. Bdph also significantly antagonized cromakalim originated relaxation. Bdph, glibenclamide and TEA did not significantly affect the antagonistic effects against cromakalim originated relaxation. All calcium channel blockers did not affect neither basal tension nor Bdph's antagonistic effect against cromakalim [32]. In our study, it was determined that APDTC continues to relax. Blocking K^{+} channels with TEA did not change the functional path of APDTC. To determine the effect of APDTC in the NANC system, 10^{-5} M phentolamine+ 10^{-6} M propranolol+ 10^{-5} M atropine (mix) were administered on the trachea smooth muscle strip. A statistically significant difference was found between ACh contraction responses in smooth muscle and the responses of APDTC in the presence of phentolamine+propranolol+atropine. APDTC created a relaxation effect in the presence of adrenergic-cholinergic receptor antagonists as mix in the tracheal smooth muscle strip contracted with acetylcholine.

In addition, the contraction effect caused by adrenergic and cholinergic receptor antagonists as phentolamine+propranolol+atropine formed a statistical relaxation effect by the administration of Ammonium Pyrrolidine Dithiocarbamate. Phentolamine+propranolol+atropine did not affect the relaxation response of APDTC to tracheal smooth muscle strip contracted with acetylcholine. When our study evaluated the effects of APDTC with proven antioxidant and anti-inflammatory effects in the trachea smooth muscle strip, the relaxation response was formed by APDTC in the trachea smooth muscle strip induced by ACh but it was not found statistically significant. We are able to suggest that different doses of APDTC can be used. The EC50 dose was not effective. Adrenergic receptors, cholinergic receptors and substances that affect L-type calcium channels and potassium channels have not changed this response, and therefore we may think that APDTC does not use adrenergic receptors, cholinergic receptors, L-type calcium channels and potassium channels. On the other hand, as a result of this research, we can argue that the NANC system, which plays an important role in tracheal smooth muscle strip contraction-relaxation responses, may contribute to find out the functions of APDTC with different in vitro studies. Recently published articles were screened to see the direction of research on the subject and these are stated as pre-clinical safety evaluation of pyrrolidine dithiocarbamate [33], effects of ammonium pyrrolidine dithiocarbamate (PDTC) on osteopontin expression and autophagy in tubular cells in streptozotocin-induced diabetic nephropathy rat [34], bidirectional effects of pyrrolidine dithiocarbamate on severe acute pancreatitis in a rat model [35], effects of local anesthetics on Smooth muscle tissue in rat trachea: an in vitro study [36], niclosamide ethanolamine induces trachea relaxation and inhibits proliferation and migration of trachea smooth muscle cells [37], design, synthesis and biological evaluation of novel ring-opened cromakalim analogues with relaxant effects on vascular and respiratory smooth muscles and as stimulators of elastin synthesis [38], effect of hydroalcoholic extract of anethum graveolens L. seed on tracheal

smooth muscle contractions in male rats [39], effects of levobupivacaine on isolated rat tracheal smooth muscle [40] and bronchodilatory effect of hydrogen sulfide in rat [41]. The future research will help to solve the unknown pieces of puzzle in asthma biology.

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ATTACHMENTS

APPENDIX 1. Experimental Animals Local Ethics Committee Approval Certificate

T.C.
DUMLUPINAR ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
ARAŞTIRMA BAŞVURUSU ONAYI

BAŞVURU BİLGİLERİ	ARAŞTIRMANIN ADI	Siçan trakea düz kası kasılma gevşeme cevapları üzerine Ammonium Pyrrrolidine Dithiocarbamate'nin etki mekanizmasının araştırılması
	ARAŞTIRMA YÜRÜTÜCÜSÜ KURUMU	Doç.Dr.M.Kasım ÇAYCI DPÜ Fen-Edebiyat Fakültesi Genel Biyoloji ABD
	PROJE YÜRÜTÜCÜSÜ KURUMU	Doç.Dr.M.Kasım ÇAYCI DPÜ Fen-Edebiyat Fakültesi Genel Biyoloji ABD
	YARDIMCI ARAŞTIRICILAR	Doç.Dr.M.Kasım ÇAYCI Yls.Oğr.Merve AKTAŞ Yls.Oğr.Merve ARAS
	ARAŞTIRMANIN TAHMİNİ SÜRESİ	12 Ay
	KULLANILACAK HAYVAN TÜRÜ VE SAYISI	Wistar Albino (E) – 70 Adet
DESTEKLEYİCİ KURULUŞ	--	

DEĞERLENDİRİLEN İLGİLİ BELGELER	Belge Adı	Tarihi
	ARAŞTIRMA BAŞVURU FORMU	16.12.2015

KARAR BİLGİLERİ	Karar No : 2015.12.06	Tarih : 23.12.2015
	Yukarıda başvuru bilgileri verilen araştırma projesi gerekçe, amaç ve yöntemler dikkate alınarak görüşüldü ve ilgili belgeler incelendi. Projenin etik açıdan uygun olduğuna, çalışmanın aşağıdaki hususlar dikkate alınarak yürütülmesine ve sorumlu araştırmacıya iletmesine OY BİRLİĞİ ile karar verildi. 1) Projede herhangi bir değişiklik gerektiğinde kurulumuzdan onay alınması, 2) Projede çalışacağı bildirilen araştırmacılarda değişiklik olduğunda kurulumuzdan onay alınması, 3) Deneysel hayvanları üzerinde yapılacak girişimin başlangıç ve bitiş tarihinin bildirilmesi, 4) Çalışma süresinde tamamlanamaz ise ek süre talebinde bulunulması, 5) Çalışma tamamlandığında sonuç raporunun gönderilmesi	

ETİK KURUL BİLGİLERİ

ÜYELER				
Unvanı / Adı / Soyadı EK Üyeligi	Uzmanlık Dalı	Kurumu	İlişki (*)	İmza
Doç.Dr. Aynur GÜLCAN Başkan	Mikrobiyoloji ve Klinik Mikrobiyoloji Anabilim Dalı	Tıp Fakültesi	<input type="checkbox"/> E <input checked="" type="checkbox"/> H	
Yrd. Doç. Dr. Ahmet KOÇAK Üye	Histoloji ve Embriyoloji Anabilim Dalı	Tıp Fakültesi	<input type="checkbox"/> E <input checked="" type="checkbox"/> H	
Yrd. Doç. Dr. Sezer AKÇER Üye	Anatomi Anabilim Dalı	Tıp Fakültesi	<input type="checkbox"/> E <input checked="" type="checkbox"/> H	
Yrd. Doç. Dr. Ceylan AYADA Üye	Fizyoloji Anabilim Dalı	Tıp Fakültesi	<input type="checkbox"/> E <input checked="" type="checkbox"/> H	
Yrd. Doç. Dr. Hasan METİNEREN Üye	Ortopedi ve Travmatoloji Anabilim Dalı	Tıp Fakültesi	<input type="checkbox"/> E <input checked="" type="checkbox"/> H	
Doç.Dr. M.Kasım ÇAYCI Üye	Biyoloji Anabilim Dalı	Fen-Edebiyat Fakültesi	<input checked="" type="checkbox"/> E <input type="checkbox"/> H	
Doç.Dr. Muhammed OYLUMLU Üye	Kardiyoloji Anabilim Dalı	Tıp Fakültesi	<input type="checkbox"/> E <input checked="" type="checkbox"/> H	
Yrd. Doç. Dr. Zülfi BAYHAN Üye	Genel Cerrahi Anabilim Dalı	Tıp Fakültesi	<input type="checkbox"/> E <input checked="" type="checkbox"/> H	
Vet. HEKİM Aydın AKCILAR Üye	Veteriner HEKİM	Tıp Fakültesi DEHYUAM	<input type="checkbox"/> E <input checked="" type="checkbox"/> H	
Yrd. Doç. Dr. Ahmet Haris Selçuk OZEN Üye	Zooloji Anabilim Dalı	Fen-Edebiyat Fakültesi	<input type="checkbox"/> E <input checked="" type="checkbox"/> H	
Erkan ERKOL Üye			<input type="checkbox"/> E <input checked="" type="checkbox"/> H	

* Araştırma ile ilişkisi