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Title Page

The extracellular cAMP - adenosine pathway in airway smooth muscle

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Running Title Page

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Nonstandard abbreviations

AC, adenylyl cyclase; AMPCP, adenosine 5′-(α,β-methylene)diphosphate; 8-Br-cAMP, 8-Bromo-cAMP; CCh, carbachol; CGS-15943, 9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine; DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine; EHNA, erythro-9-(2-Hydroxy-3-nonyl) adenine hydrochloride; Gi, inhibitory G protein; IBMX, 3-isobutyl-1-methylxanthine; TR-FRET, time-resolved fluorescence resonance energy transfer.

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Abstract

In the respiratory tract, intracellular cAMP has a key role in the smooth muscle relaxation induced by β_2-adrenoceptors/Gs protein/adenylyl cyclase axis. In other tissues, cAMP also works as an extracellular messenger, after its efflux and interstitial conversion to adenosine by ectoenzymes. The aim of this study was to identify cAMP efflux and "extracellular cAMP-adenosine pathway" in the airway smooth muscle. Firstly, we tested the ability of β_2-adrenoceptor agonists formoterol or fenoterol to increase the extracellular cAMP in isolated tracheal rings from adult male Wistar rats. The effects of adenosine, cAMP, 8-Br-cAMP, fenoterol or formoterol were also evaluated in the isometric contraction of control or carbachol (CCh) precontracted tracheas, normalized as percentage of CCh-induced response. Fenoterol and formoterol induced 70-80% relaxation and increased by up to 280-450% the extracellular cAMP levels. While exogenous cAMP or adenosine evoked phasic contractions, the membrane-permeable cAMP analog 8-Br-cAMP induced relaxation of CCh-precontracted tracheas. The simultaneous inhibition of adenosine degradation/ uptake with EHNA plus uridine increased by 3-fold the maximum cAMP-induced contraction, whereas it was significantly reduced by AMPCP (ecto-5'-nucleotidase inhibitor), and by adenosine receptor antagonists CGS-15943 (non-selective) or DPCPX (A_1-selective). Finally, CGS-15943 shifted to the left the concentration-relaxation curve for fenoterol. In conclusion, our results show that airway smooth muscle expresses the "extracellular cAMP-adenosine pathway" associated with contracting effects mediated by A_1 receptors. The cAMP efflux triggered by fenoterol/formoterol indicates that the extracellular cAMP-adenosine pathway may play a role in balancing relaxant effects of β_2-adrenoceptor agonists in airways, which may impact their bronchodilation effects.

Introduction

Adenosine is an endogenous purine nucleoside that works as modulator of numerous cellular and molecular functions via activation of four adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) coupled to G protein (GPCR). It is well established that ATP, substrate for ectonucleotidases (CD39 and CD73) serves as important source of extracellular adenosine (Fredholm, et al., 2011). However, since the middle 1990s, when the extracellular cAMP-adenosine pathway was first described in mammalian tissue (Mi and Jackson, 1995), a correlation between cAMP efflux through ABCC transporters and its degradation by ectoenzymes have been pointed as an alternative source of extracellular adenosine (Mi and Jackson, 1995; Jackson and Raghvendra, 2004). This metabolic system has become focus for an extracellular feedback mechanism that allows the control of physiological responses triggered by activation of adenylyl cyclases (AC). In fact, several studies have reported the existence of the extracellular cAMP-adenosine pathway in mammalians cells, tissues and organs (revised in Godinho *et al.*, 2015). In this respect, previous findings from our lab have shown that activation of β_2-adrenoceptor/ Gs protein/AC axis triggers the cAMP efflux from skeletal muscle cells (Godinho and Costa-Jr, 2003; Chiavegatti, et al., 2008) that exerts an extracellular negative feedback effect on muscle contraction through adenosine formation and activation of postsynaptic A_1 receptors (Duarte, et al., 2012).

Taking into account a) the central role of β_2-adrenoceptors/ AC/ cAMP signaling cascade in airway smooth muscle relaxation (Cazzola, et al., 2012), b) the autocrine or paracrine function of cAMP egress in skeletal, cardiac and smooth muscles (Godinho and Costa-Jr, 2003; Cheng, et al., 2010; Sassi, et al., 2011), c) the increased release of adenosine from airway cells during inflammatory processes associated to chronic lung disease (Huszar, et al., 2002; Adriaensen and Timmermans, 2004), and d) the growing evidence for

adenosine involvement in bronchoconstriction in asthmatic patients (Wilson, et al., 2009), we hypothesize that airway smooth muscle would exhibit cAMP efflux in response to β_2-adrenoceptor agonists and the extracellular cAMP–adenosine pathway, which could play a regulatory role in the airway smooth muscle contraction.

To test this idea, the effects of β_2-adrenoceptor agonists were evaluated on the extracellular cAMP level in an attempt to identify the efflux of cAMP from rat tracheal tissue. In addition, we also investigated the effect of exogenous cAMP on the isometric contraction of the rat isolated trachea under inhibition of a) both adenosine deaminase and adenosine uptake, with EHNA plus uridine, b) ecto-5'-nucleotidase, with AMPCP, c) adenosine receptors with the antagonists of adenosine receptors CGS-15943 (non-selective) or DPCPX (A_1 selective).

Our results showed that stimulation of airway smooth muscle with β_2-adrenoceptor formoterol and fenoterol induces tracheal smooth muscle relaxation, which is accompanied by cAMP efflux. Outside the tracheal cells, the cAMP is able to induce contracting effects mediated by activation of A_1 receptors. These results indicate that the extracellular cAMP-adenosine pathway may play a role in balancing relaxant effects of β_2-adrenoceptor agonists in airway smooth muscle, which may affect the efficacy of these bronchodilators.

Methods

Animals and ethical approval

Adult male Wistar rats (3 to 4-month-old; 250-350 g) were obtained from the Laboratory of Animal Experimentation of the National Institute of Pharmacology and Molecular Biology (LEA-INFAR), UNIFESP, SP, Brazil. All animals were maintained in pathogen-

free environment under controlled conditions (22 ± 2°C and 50 ± 15% relative humidity) in a 12 h light/dark cycle with free access to food and water. Rats were housed in clear polyethylene cages (4 per cage) under standard laboratory conditions. The animal procedures and experimental protocols were approved by the Ethic Committee on Animal Use of the Federal University of São Paulo (CEUA/UNIFESP, protocol #9987150714). Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Isolated tracheal ring preparation

Rats were killed by decapitation and trachea, heart and lungs were excised *en bloc*. The whole trachea was dissected and carefully cleaned from connective tissues. Two isolated tracheal segments containing four to five cartilaginous rings were obtained from the distal portion of trachea (above the carina) (de Lima and da Silva, 1998) and immediately mounted in an isolated organ bath system (AVS Projetos, São Carlos, Brazil). The tracheal segments were positioned horizontally and suspended between stainless steel wire hooks in the organ bath containing salt buffer solutions at 37°C, pH 7.4 and continuously gassed with 95% O_2/5% CO_2. The lower hook was attached to a fixed holder at the bottom of the organ bath, and the upper hook was attached to an isometric force transducer (FT.03, Grass Instruments, USA) connected to a computer data acquisition system (PowerLab, AD Instruments, Australia). The tracheal rings were gently stretched under a basal tension of 9.80 mN and equilibrated for 1 h before beginning of the experiments.

Isometric contraction studies

After the equilibration period, the trachea segments were exposed to 1 μM carbachol (CCh) for 2 min and washed several times with Krebs solution, over a period of 30 min, to ensured muscle relaxation to a basal tension and achievement of reproducible contractions. After 1 h, the effects of drugs that interfere with the cAMP-adenosine pathway were investigated on tracheal contraction using two different experimental protocols: (i) the isolated tracheal rings under basal tension or (ii) CCh-precontracted isolated tracheal rings. The isometric contractile forces were collected and analyzed using LabChart 7 software (ADInstruments, Australia). The Krebs's solution (5-mL organ bath) was employed in the contraction studies and consisted of: 119 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$ and 11 mM glucose, pH 7.4). The values of the contractile responses were normalized as percentages of maximal response obtained with 1 μM CCh. Relaxation responses were normalized as percentages of response obtained with EC_{30} of CCh. Concentration-response curves were analyzed by nonlinear regression (GraphPad Prism 5, Graph Pad Software, San Diego, USA) using the three-parameter logistic. The values of potency (pEC_{50}) and maximal response (E_{max}) were obtained from concentration-response curves.

Effect of cAMP and adenosine on tracheal rings under basal tension

In the first experimental protocol, the tracheal rings were exposed to 1 μM CCh and the effects of 300 μM cAMP or 100-300 μM adenosine were examined on the contractility of isolated rat tracheal rings immediately after the stabilization of basal tension. In some experiments, the amplitude of contraction induced by cAMP was analyzed in tracheal rings pretreated 60 min with AMPCP (100 μM; inhibitor of ecto-5'-nucleotidase), CGS-15943 (20 μM; a nonselective adenosine receptor antagonist), a cocktail containing

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EHNA (10μM; inhibitor of adenosine deaminase) and uridine (50 μM; inhibitor of adenosine uptake), or their respective vehicles.

Effect of cAMP, adenosine and β_2-agonist on tracheal rings precontracted with carbachol

In a group of experiments, another set of experiments, after the stabilization of basal tension, a cumulative concentration–response curve to CCh was obtained to find the concentration of CCh that produces 30% of the maximal response (CCh EC_{30}). Thereafter, the tracheas were stimulated with the CCh EC_{30} and contractile responses were recorded for 10 min. After 1 h washout out of the agonist and restoration of the basal tension, the tracheas were precontracted again with the CCh EC_{30} for 10 min, and incubated with formoterol (1 μM; long-acting β_2-agonist), fenoterol (1 μM; short-acting β_2-agonist), 8-Br-cAMP (100 μM; cell-membrane permeable cAMP analogue) and compared to those of 300 μM of cAMP.

In another set of experiments, tracheal segments precontracted with CCh EC_{30} were incubated with increasing non-cumulative concentrations of adenosine (1-1000 μM) or cAMP (1-1000 μM) in the presence or absence of EHNA (10 M) plus uridine (50 μM). In a further sequence of experiments, the influence of 100 μM AMPCP, 2 μM CGS-15943 or 100 μM DPCPX was evaluated on the cAMP-induced contraction of tracheal rings precontracted with CCh. Inhibitors and competitive antagonists were added to the organ bath 60 min before cAMP incubation.

Effect of non-selective adenosine receptor antagonist on fenoterol-induced relaxation of carbachol-precontracted tracheal rings

Tracheal preparations were precontracted with CCh EC_{30} for 10 min and then the first cumulative concentration–response curve to fenoterol was constructed by adding increasing concentrations of the agonist (10^{-9} M – 10^{-4} M). Following a 10 min washout period, the tissues were incubated with CGS-15943 2 µM or vehicle (DMSO 0.2%) for 60 min, precontracted with CCh EC_{30} for 10 min and then a second concentration–response curve to fenoterol was obtained. Finally, after washing and precontraction of the tracheal rings with CCh EC_{30} for 10 min, a third concentration–response curve to fenoterol was obtained in the presence of CGS-15943 20 µM or vehicle (DMSO 0.2%) previously incubated for 60 min.

Measurement of extracellular cAMP

The experiments were performed using Tyrode's solution, (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 15 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2 mM $CaCl_2$ and 11 mM glucose, pH 7.4), which have been stablished as the standard buffer solution for determination of cAMP content in skeletal and smooth muscles in our laboratory. After 1 h equilibration period in an organ bath containing Tyrode's solution, the tracheal rings were incubated with 1 mM IBMX (nonselective inhibitor of intra- and extracellular PDE) for 30 min in order to inhibit intra- and extracellular degradation of cAMP, and then stimulated with 1 µM formoterol or 1 µM fenoterol. The incubation medium was collected at 0, 10, 30 and 60 min following agonist treatment, transferred into microtubes containing ice-cold EDTA (4 mM final concentration) and immediately boiled in a dry bath for 15 min, in order to denaturate PDEs and prevent cAMP hydrolysis. The samples were centrifuged at 10.000 x *g* for 15 min at 4°C (Chiavegatti, et al., 2008) and determination of cAMP levels from supernatants were performed using the Lance Ultra cAMP Kit (Perkin Elmer, Waltham, USA) according to the manufacturer's instructions, in 96-well half-area

microplates (Perkin Elmer, Waltham, USA). The time-resolved fluorescence resonance energy transfer (TR-FRET) signal was measured using Flex Station 3 (Molecular Device, USA) with excitation at 340 nm and emission at 615 nm and 665 nm, after a delay time of 50 μs and integration time of 100 μs. The levels of extracellular cAMP were expressed as pmol·per tissue mass (pmol/ mg).

Data and statistical analysis

The data obeyed the normal distribution, i.e., they have passed the Kolmogorov–Smirnov test under the significant level of $\alpha = 0.05$ and were presented as mean ± standard error of mean (SEM) and experimental number (n) represents the number of trachea segments obtained from different rats. Statistical analyses were performed using GraphPad Prism software (version 5.01; Graph- Pad Software Inc., San Diego, CA). Differences between groups were determined by Student's *t* test or one-way ANOVA followed by Dunnett's multiple comparison test, and considered significant at $P < 0.05$

Materials

8-Bromo-cAMP sodium salt, EHNA hydrochloride and formoterol hemifumarate were purchased from Tocris Bioscience (Ellisville, MO, USA). Adenosine, carbachol (carbamoylcholine chloride), cyclic AMP (adenosine 3′,5′-cyclic monophosphate), CGS-15943 (9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine), DPCPX (8-Cyclopentyl-1,3-dipropylxanthine; fenoterol (fenoterol hydrobromide), uridine, AMPCP (adenosine 5′-(α,β-methylene)diphosphate) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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Results

β_2-adrenoceptor agonists induce relaxation of tracheal smooth muscle and stimulate extracellular cAMP accumulation

We have first investigated whether relaxation induced by activation of rat tracheal β_2-adrenoceptors could be followed by cAMP efflux. As shown in Figure 1A, β_2-adrenoceptor agonists fenoterol (1 µM, short-acting) and formoterol (1 µM, long-acting) relaxed CCh-precontracted tracheas by 79% and 69%, respectively. The relaxing effects of these β_2-adrenoceptors agonists last for at least 30 min (data not shown) and were accompanied by a gradual increase in extracellular cAMP levels that reached 553% and 383% of the basal values (time zero: fenoterol = 1.52 ± 0.24 pmol/ mg tissue, formoterol = 1.83 ± 0.42 pmol/ mg tissue), after 60 min stimulation (Figure 1B). A discrete but significant increase (75%) in the extracellular cAMP was also found in control tracheas treated for 60 min with vehicle (time zero: vehicle =1.59 ± 0.30 pmol/ mg tissue; Figure 1B), indicating that a basal cAMP efflux occurs even in the absence of β_2-adrenoceptor activation.

Extracellular cAMP induces contraction of rat precontracted trachea via activation of A_1 adenosine receptors by its metabolite adenosine

To examine the possible functional relevance of extracellular cAMP on the airway smooth muscle contraction, we compared the effect of the exogenous 3',5'-cAMP, which is unable to enter the cell (Robison, et al., 1965), with that of 8-Br-cAMP, a cell-membrane permeable cAMP analogue that is resistant to intra- and extracellular PDEs. As shown in Figure 2, while 300 µM cAMP caused contraction of tracheal smooth muscle

(Figure 2A and C), the 8-Br-cAMP relaxed by 43% the CCh-precontracted tracheal rings (Figure 2 B and C).

The contracting effect of cAMP was mimicked by adenosine (Figure 3). However, the amplitudes of contractile responses induced by either cAMP (Figure 3A) or adenosine (Figure 3B) in CCh-precontracted tracheal rings were ~2.5-fold greater than those under basal conditions.

In fact, incubation of CCh-precontracted tracheal rings with increasing concentrations of cAMP (3-1000 µM) elicited phasic contractions, in a concentration-dependent manner (Figure 4A and B). A similar effect was observed with adenosine (1-1000 µM) (Figure 4C and D). However, the potency (pEC_{50}) of adenosine, obtained from the analysis of non-cumulative concentration-response curves, was 11-fold greater than that of cAMP (Figure 4G; Table 1). In addition, the E_{max} for adenosine was significantly higher than that seen for cAMP (Table 1).

In order to evaluate the contribution of the metabolite adenosine to the contractile effect of extracellular cAMP in the rate tracheal rings, we inhibited the adenosine deaminase and adenosine uptake with a cocktail containing EHNA and uridine. In these conditions, cAMP elicited concentration-dependent phasic contractions (Figure 4E and F) with an E_{max} ~3 fold higher than in the absence of the cocktail inhibitors (Figure 4G; Table 1). No significant change in the potency of cAMP was observed in the presence of EHNA and uridine (Figure 4G; Table 1). Representative records of isometric contraction elicited by 300 µM cAMP, 300 µM adenosine or EHNA/uridine plus 300 µM cAMP in CCh-precontracted tracheal rings are shown in Figure 4B, D and F.

Finally, we investigated the involvement of ecto-5'-nucleotidase and adenosine receptor in the contractile response elicited by extracellular cAMP. For these experiments, rat tracheal rings were preincubated for 60 min with 100 µM AMPCP (inhibitor of ecto-5'-

nucleotidases), 2 μM CGS-15943 (non-selective adenosine receptor antagonist) or 100 nM DPCPX (selective A_1 adenosine receptor antagonist) before addition of 300 μM cAMP. As seen in Figure 5A, preincubation of the tracheas with AMPCP reduced by 57% the cAMP-induced contraction. Likewise, pre-incubation of tracheas with CGS-15943 or DPCPX reduced by 63% and 54% the contraction induced by cAMP (Figure 5B and C).

Extracellular cAMP induces contraction of rat trachea under basal tonus

As observed in precontracted trachea segments, under basal tonus the amplitude of contraction induced by adenosine 300 μM was higher than that observed with the same concentration of cAMP (Figure 6A). Pretreatment of smooth muscle preparation with EHNA/uridine increased by 2-fold the contractile effect of 300 μM cAMP (Figure 6B). Finally, cAMP-induced contraction was almost completely abolished by preincubation of tracheal preparation either with the ecto-5'-nucleotidase inhibitor AMPCP (Figure 6C) or with the non-selective adenosine receptor antagonist CGS-15943 (Figure 6D).

Non-selective adenosine receptor antagonist potentiates the relaxation response induced by β_2-adrenergic receptor agonist

In order to evaluate the reproducibility of the cumulative concentration–response to fenoterol in the same tracheal preparations, we initially constructed three consecutive concentration–response curves to fenoterol at intervals of 60 min. As shown in Figure 7A, the first concentration–response curve to fenoterol presented a pEC_{50} of 7.03 ± 0.18 (n=4). However, second and third concentration–response curve to fenoterol produced a 5-fold (pEC_{50} second curve = 6.36 ± 0.10; n=4) and 11-fold rightward shift (pEC_{50} third curve = 5.99 ± 0.13; n=4), respectively. The reduction in fenoterol potency observed after consecutive concentration–response curves forced us to evaluate the effect of adenosine

receptor antagonist CGS-15943 and its vehicle in different tracheal segments of the same rat. As shown in figure 7B, the first concentration–response curve to fenoterol obtained in different tissues exhibited similar potencies. Pretreatment of tracheal rings with 2 μM CGS-15943 induced a 2-fold leftward shift of the second concentration–response curve to fenoterol without reduction in the maximal relaxation response (Figure 7C). At 20 μM, CGS-15943 induced a 11-fold leftward shifts ($P < 0.05$; Student's t-test) in the third fenoterol concentration–response curve without affecting the maximal relaxation (Figure 7D). The values of pEC_{50} to fenoterol obtained in tracheas treated with CGS-15943 or vehicle are shown in Table 2.

Discussion

The extracellular cAMP-adenosine pathway has been described in several mammalian tissues, functioning as an extracellular feedback mechanism triggered in response to changes in intracellular cAMP levels (Jackson and Raghvendra, 2004; Godinho, et al., 2015). Although the cAMP efflux from isolated perfused rat lung has been mentioned by Barnard (1994) and recently described in human airway epithelial cells (Huff, et al., 2017), its biological function in the airway smooth muscle has never been experimentally addressed.

The current study provides reliable evidence for a functional extracellular cAMP-adenosine pathway in airway smooth muscle. Firstly, the long-acting β_2-adrenoceptor agonist formoterol, used as bronchodilator for the management of persistent asthma symptoms, evoked a time-dependent efflux of cAMP from isolate tracheal rings, which was mimicked by the short-acting β_2 agonist fenoterol (Figure 1). These results show that, in tracheal smooth muscle, β_2-adrenoceptor-induced increase in intracellular cAMP formation is followed by the efflux of the cyclic nucleotide to the extracellular space.

Interestingly, the exogenous cAMP was able to induce airway smooth muscle contraction (Figure 2A), likely through an extracellular mechanism, since cell membrane is impermeable to cAMP (Robison, et al., 1965). Considering the wet weight of tracheal tissue used in the present study (26-28 mg), the cAMP released in 2,5 ml of medium (~ 40-130 nM) after β_2-adrenoceptors stimulation, and the extracellular fluid volume of rat trachea (~ 1 ml/ g of dry tissue weight) (Woie and Reed, 1997), the cAMP released by the tracheal preparation (figure 1B) could reach micromolar extracellular levels, which is the concentration required to induce muscle contraction (figure 2A).

Actually, the opposite effect of membrane-permeable cAMP analog 8-Br-cAMP (Figure 2B) and the significant reduction of cAMP-induced contraction observed in tracheal segments pre-treated with ecto-5'-nucleotidases inhibitor AMPCP (Figures 5 A and 6C) support an extracellular site of action of exogenous cAMP. The required involvement of ecto-5'-nucleotidase also indicates that the effect of cAMP on tracheal contractility depends on the extracellular cyclic nucleotide degradation into adenosine. Besides, while cAMP-induced contraction has been inhibited by non-selective (CGS-15943) and A_1 selective (DPCPX) adenosine receptor antagonists (Figures 5B-C and Figure 6D), it was oppositely affected by adenosine deaminase and adenosine uptake cocktail inhibitors (Figures 4E-G and 6B), showing that the effect of exogenous cAMP on tracheal contractility involves the activation of A_1 adenosine receptors, through its metabolite adenosine as outlined in Figure 8.

Our results also show that the contracting effect of extracellular cAMP is intensified in tracheal muscle pre-contracted with the cholinergic agonist CCh (Figure 3), indicating a synergistic effect of AMPc/adenosine and carbachol on the airway smooth muscle contraction. Essentially, these synergistic effects are consistent with the net increase in the intracellular Ca^{2+} induced by both CCh, via activation of smooth muscle muscarinic

m3 receptors/ Gq protein (Wang and Kotlikoff, 1997; Billington and Penn, 2002), and adenosine, via A_1 adenosine receptors/ Gi protein (Gerwins and Fredholm, 1992; Abebe and Mustafa, 1998).

Increasing evidence suggest that adenosine may be important mediator of bronchial asthma since its levels are elevated in the bronchoalveolar lavage fluid of asthma patients (Driver, et al., 1993). Using ragweed sensitized and challenged mice as an allergic animal model, Fan and Mustafa (2002) showed that inhalation of adenosine causes a dose-related bronchoconstriction, which was associated with enhanced influx of inflammatory cells into the bronchoalveolar lavage fluid. In addition, Yip et al., (2011) showed a inhibitory effect of A_1 receptor antagonists on human mast cell activation. Adenosine also induces histamine release from human bronchoalveolar lavage mast cells (Forsythe, et al., 1999). Furthermore, it is well known that at least part of the bronchodilator and anti-inflammatory effects of theophylline has been linked to adenosine receptors inhibition (Fredholm and Persson, 1982; Cheng, et al., 2017). Actually, our results showed that inhibition of adenosine receptors with CGS-15943 shifted to the left the relaxing curve of fenoterol (figure 7C-D). The ability of adenosine receptor antagonist to potentiate the relaxing effect of fenoterol effect suggests that combining a beta-2 receptor agonist with a selective A_1 receptor blocker might provide better clinical control of lung diseases (asthma and COPD). Therefore, taking into account the efflux of cAMP from tracheal cells and its extracellular degradation into adenosine, it is reasonable to suppose that the extracellular cAMP-adenosine pathway may influence airway function in diseases such as asthma. In fact, the existence of the extracellular cAMP – adenosine pathway in tracheal smooth muscle may influence the bronchodilator effects of β_2 adrenoceptor agonists, which is under investigation in our lab in animal models of airway inflammatory and allergic diseases.

Taken together, our results show that β_2-adrenoceptor induces cAMP efflux from tracheal smooth muscle which is consistent with previous observations showing that activation of β_2-adrenoceptor by isoprenaline stimulates cAMP efflux in human airway epithelial cell (Geary, et al., 1993) and rat lung perfusate (Barnard, et al., 1994).

More importantly, to our knowledge our study is the first to report the presence of the extracellular cAMP-adenosine pathway in airway smooth muscle. Moreover, using pharmacological strategies we also show that the interstitial cyclic AMP is metabolized by ectoenzymes into adenosine, which induces tracheal smooth muscle contraction by activating adenosine receptors. Considering the synergistic effects of extracellular cAMP and CCh, that mimics the increased smooth muscle tone observed in asthma, the impact of cAMP efflux and the extracellular cAMP-adenosine pathway in the animal model of allergen-induced asthma is also under investigation by our group.

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Authorship Contributions

Participated in research design: ES Pacini and RO Godinho

Conducted the experiments: ES Pacini and S Sanders-Silveira

Performed data analysis: ES Pacini and RO Godinho

Wrote or contributed to the writing of the manuscript: ES Pacini and RO Godinho

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Footnotes

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Legends For Figures

Figure 1. Relaxing effects of β_2-adrenoceptor agonists in rat tracheal rings is followed by increment in extracellular cAMP levels. (A) Maximum relaxation induced by 1 µM formoterol or 1 µM fenoterol in rat tracheal rings precontracted with CCh EC_{30} (n = 5-8). (B) Time course of extracellular cAMP accumulation in isolated tracheal rings exposed to 1 µM formoterol or 1 µM fenoterol or vehicle (n = 5-6) in the presence of 1 mM IBMX. Data are presented as mean ± SEM. *Significantly different from control group (Vehicle) ($P < 0.05$; Student's t-test).

Figure 2. cAMP and 8-Br-cAMP induce opposite inotropic effects in rat tracheal rings precontracted with carbachol (CCh) EC_{30}. Representative records of isometric contractions induced by 300 µM cAMP (A, cell membrane non-permeable cyclic nucleotide) and 100 µM 8-Br-AMPc (B, cell-membrane permeable cAMP analog). (C) Data presented as mean ± SEM and normalized as percentage of the response induced by CCh EC_{30} (n = 5-10). *Significantly different from control group (Vehicle) ($P < 0.05$; Student's t-test).

Figure 3. Adenosine mimics the positive inotropic effect of cAMP in resting and precontracted rat tracheal rings. Maximum contraction induced by 300 µM cAMP (A; n = 10-11) or 100 µM adenosine (B; n = 5-6) in rat tracheal rings under basal tonus (white bar) or precontracted with CCh EC_{30} (dashed bar). Data are presented as mean ± SEM and normalized as percentage of the response induced by 1 µM carbachol. *Significantly different from tracheal rings under basal tonus ($P < 0.05$; Student's t-test).

Figure 4. Simultaneous inhibition of adenosine deaminase and adenosine uptake increases the contracting effect of cAMP. The isolated rat tracheas were contracted with CCh EC_{30} for 10 min and the contractile response induced by non-cumulative addition of cAMP (A; n = 5-20), adenosine (C; n = 5-8) or cAMP plus EHNA and uridine (F; n = 5-7) were observed. Representative records of isometric contractions induced by 300 µM cAMP (B), 300 µM adenosine (D) and 300 µM cAMP plus EHNA and uridine (F). Concentration-response curves derived for each drug in precontracted rat trachea (G). Data are presented as mean ± SEM. *Significantly different from contraction induced by 1 µM CCh ($P < 0.05$; ANOVA followed by Dunnett's multiple comparison test).

Figure 5. Inhibition of either ecto-5'-nucleotidase or A_1 adenosine receptors reduces the cAMP-induced contractile response in rat tracheal rings precontracted with CCh EC_{30}. Isolated rat tracheas were incubated with AMPCP, a selective ecto-5'-nucleotidase inhibitor (A; n = 6), CGS-15943, a non-selective adenosine receptor antagonist (B; n = 5) or DPCPX, a selective A_1 adenosine receptor antagonist (C; n = 7) for 60 min, precontracted with CCh EC_{30} and the contractile response induced by cAMP was observed. Data are presented as mean ± SEM. *****Significantly different from 300 µM cAMP ($P < 0.05$; Student's t-test).

Figure 6. Pharmacological characterization of extracellular cAMP-adenosine pathway in rat tracheal rings under basal tonus. After stabilization of basal tonus, the tracheal rings were stimulated with cAMP or adenosine (A; n = 7-11) and the amplitude of contraction were measured. The contractile effect promoted by cAMP was also evaluated in the presence of uridine plus EHNA (B; n = 9), AMPCP (C; n = 3) or CGS15943 (D; n = 4).

Data are presented as mean ± SEM. *Significantly different from 300 μM cAMP ($P < 0.05$; Student's t-test).

Figure 7. Adenosine receptor antagonist potentiates the fenoterol-induced relaxation response in rat tracheal rings precontracted with CCh EC_{30}. Three cumulative concentration-response curves to fenoterol were constructed at 60 min intervals (A, n=4). Control cumulative concentration-response curves to fenoterol from different tissues (B, n=4-5). Isolated rat tracheas were incubated with vehicle (DMSO 0.2%) or CGS-15943 2 μM (C; n = 4-5) and 20 μM (D; n = 3-4) for 60 min, precontracted with CCh EC_{30} and the cumulative concentration-response curves to fenoterol were constructed. Data are presented as mean ± SEM.

Figure 8. Model of extracellular cAMP – adenosine signaling in airway smooth muscle. Activation of β_2-adrenoceptor induces a Gs-dependent activation of adenylyl cyclase and cAMP-dependent relaxation of smooth muscle. Part of intracellular cAMP is transported to the extracellular space, where it is sequentially converted to AMP and adenosine by the ectoenzymes. Then, the extracellular adenosine is able to activate A_1 adenosine receptors inducing a secondary contraction of the smooth muscle.

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Tables

Table 1. Values of potency (pEC_{50}) and maximal response (E_{max}) for cAMP, adenosine and cAMP plus EHNA and uridine obtained in rat tracheal rings precontracted with carbachol EC_{30}.

	cAMP	Adenosine	cAMP + EHNA+uridine
E_{max} (%)	18,76 ± 5,66	59,58 ± 1,27*	56,64 ±12,80*
pEC_{50}	3,71 ± 0,51	4,79 ± 0,06*	3,83 ± 0,36

A non-cumulative concentration-response curve for cAMP, adenosine and cAMP plus EHNA and uridine were constructed from data demonstrated in Figure 4 and analyzed through a nonlinear regression with GraphPad Prism 5 Software. Values are presented as mean ± SEM (n = 5-20). *****Significantly different from cAMP ($P < 0.05$; Student's t-test).

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Table 2. Values of potency (pEC50) obtained for fenoterol in the absence and presence of CGS-15943 in rat tracheal rings precontracted with carbachol EC30.

Treatment	pEC_{50} (n)		
	First Curve	Second Curve	Third Curve
Fenoterol (segment 1)	7.03 ± 0.18 (4)		
Fenoterol (segment 2)	7.20 ± 0.14 (5)		
+ Vehicle (segment 1)		6.36 ± 0.10 (4)	
+ CGS 2 µM (segment 2)		6.61 ± 0.17 (5)	
+ Vehicle (segment 1)			5.99 ± 0.13 (4)
+ CGS 20 µM (segment 2)			7.04 ± 0.32***** (3)

Three consecutive cumulative concentration-response curves for fenoterol were constructed using 2 tracheal preparations (segments 1 and 2) of the same rat (figure 7) and analyzed through a nonlinear regression with GraphPad Prism 5 Software. Values are presented as mean ± SEM . *Significantly different from vehicle (third curve) ($P < 0.05$; Student's t-test).

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Figures

Figure 1

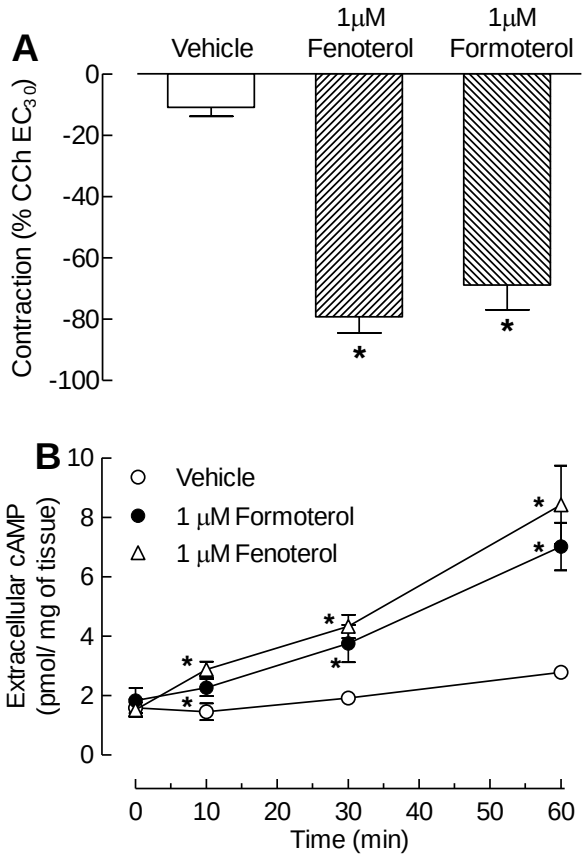

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Figure 2

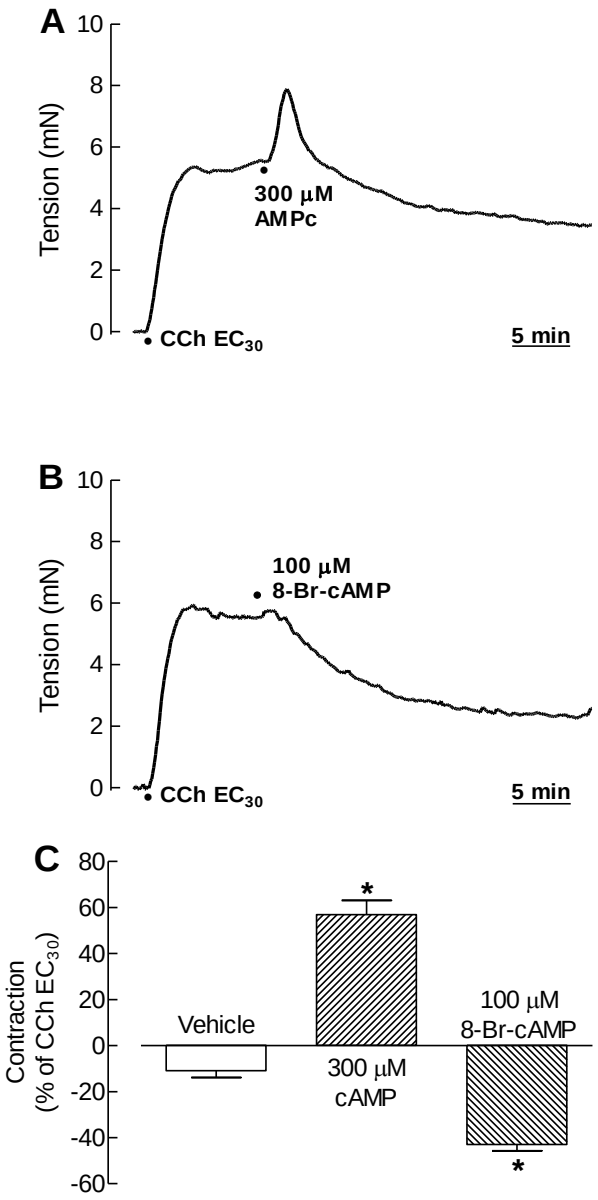

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Figure 3

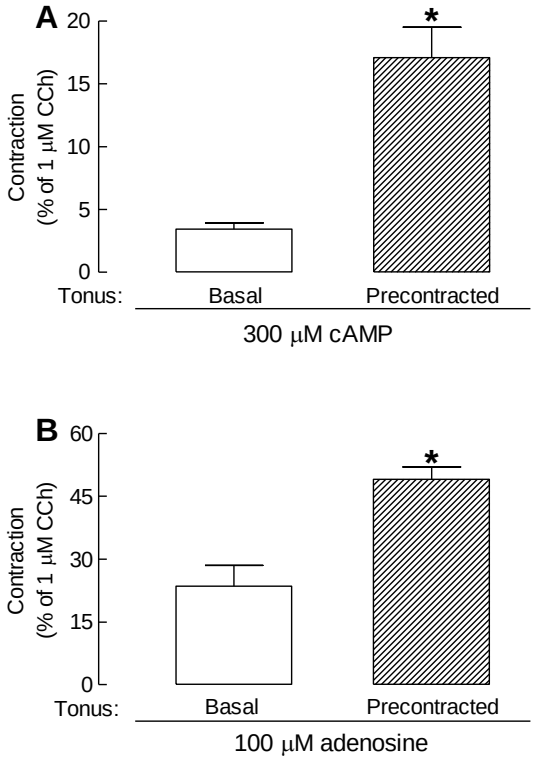

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Figure 4

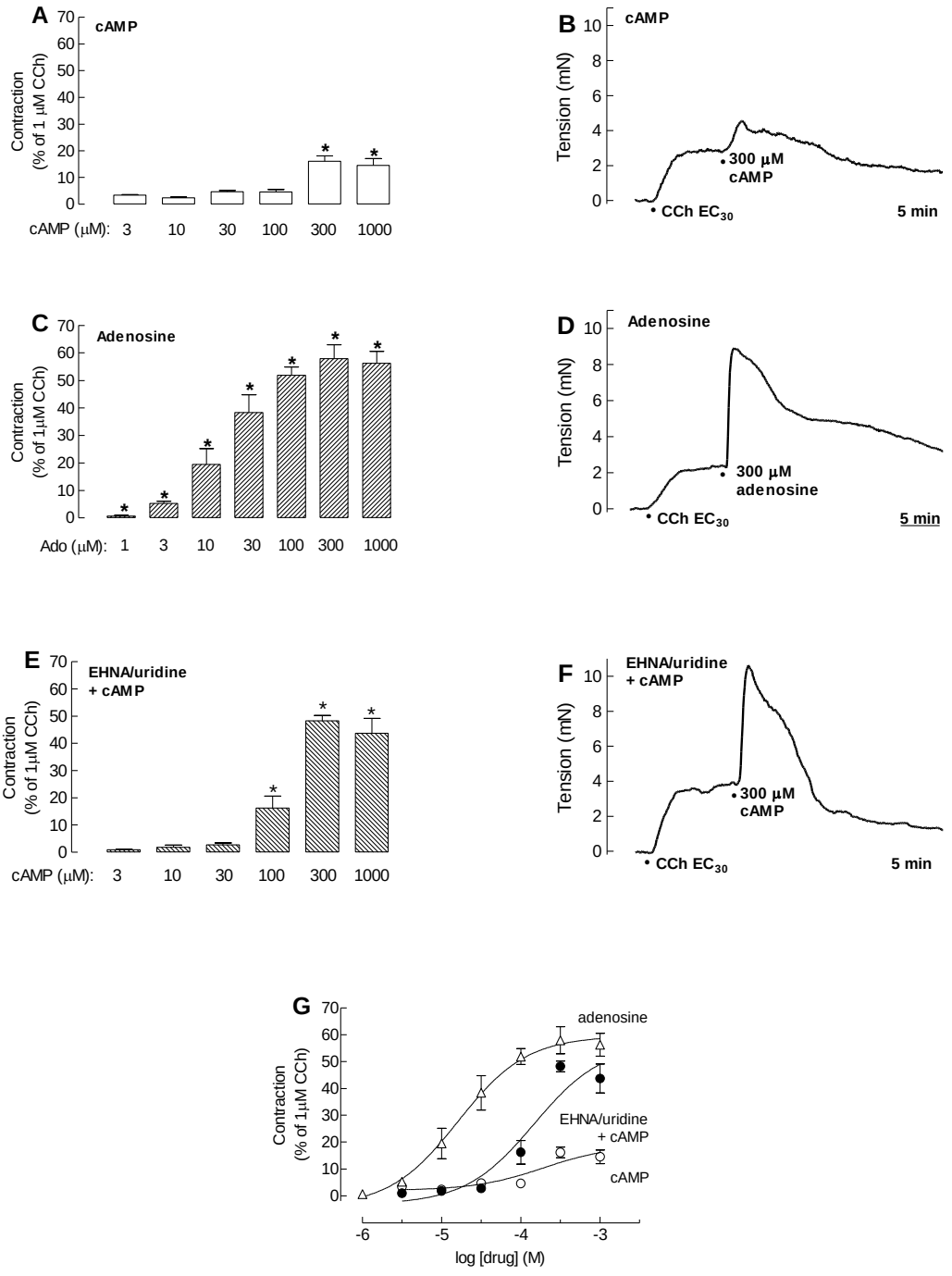

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Figure 5

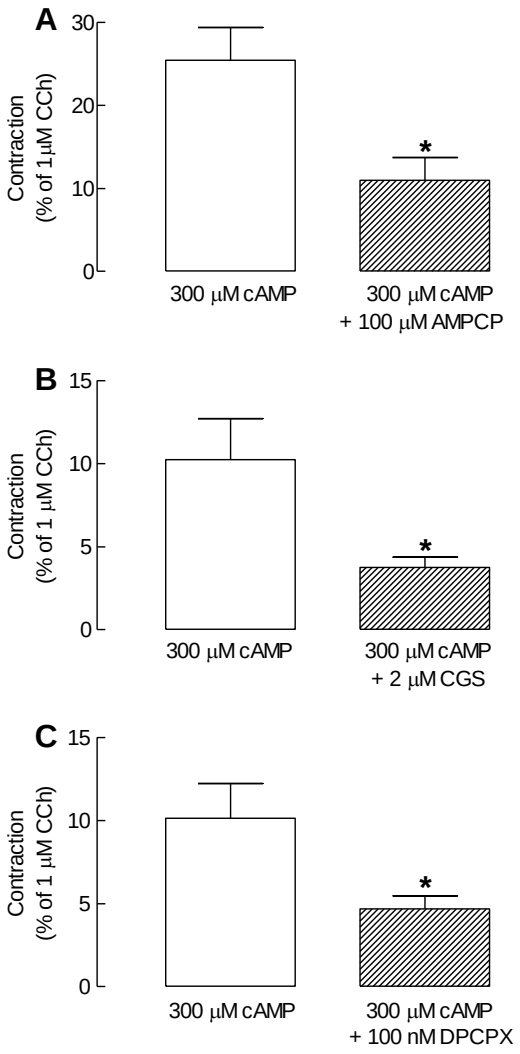

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Figure 6

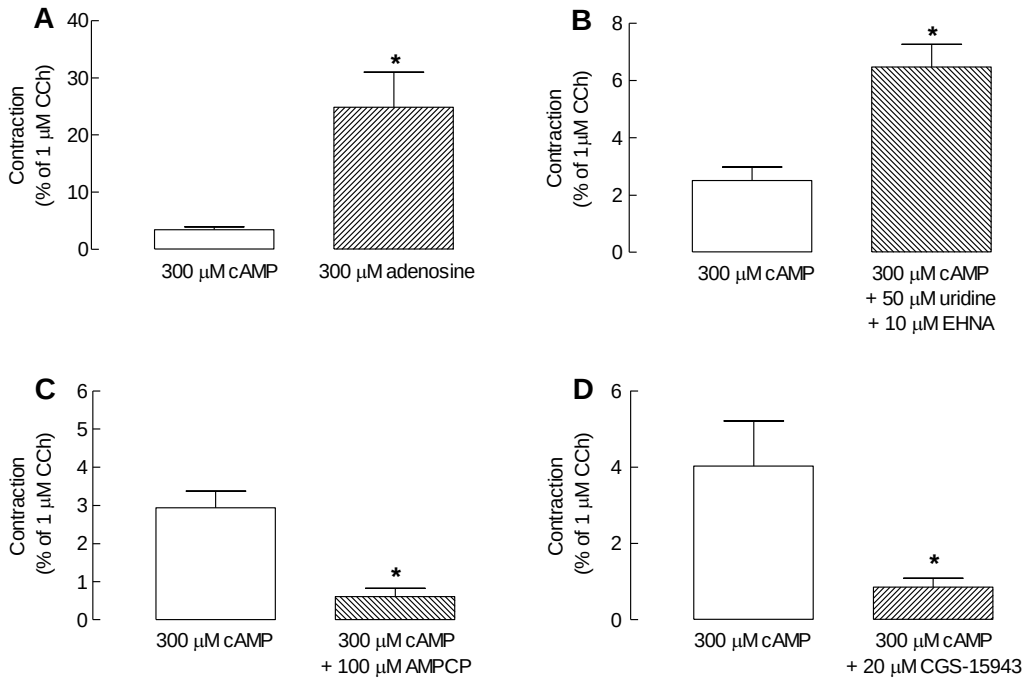

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Figure 7

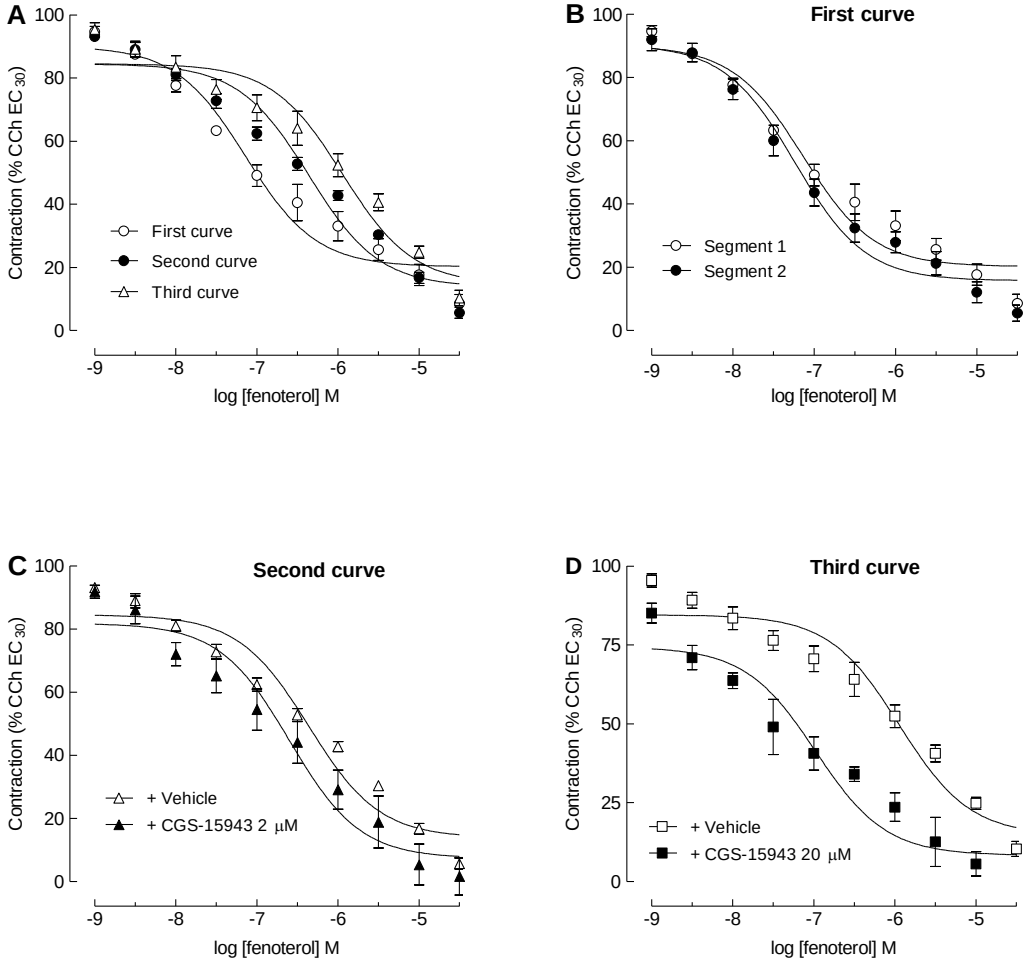

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Figure 8