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Original article

Effect of Roflumilast in airways disorders via dual inhibition of phosphodiesterase and Ca^{2+}-channel

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ABSTRACT

The bronchodilator effects of Roflumilast "a selective phosphodiesterase type-4 (PDE4)" inhibitor studied in this experimental protocol. The spiral strips of isolated guinea-pig tracheal chains mounted in organ bath and maintained in Krebs solution ventilated with carbogen at 32 °C and in Ca^{++} restricted krebs solution. PDE inhibitory activity was evaluated by recording dose response curves using inhibitory effect of isoprenaline on CCh induced contractions. For confirmation of PDE inhibition the intracellular cAMP levels were also estimated. Roflumilast resulted a sharp inhibition in contractile responses of carbachol (CCh, 1 μM) and K^+ (80 mM) and the results were almost similar to verapamil. In Ca^{++} restricted Krebs solution, a rightward shift in the Ca^{++} response curves observed in the tracheal chain strips which were pretreated with Roflumilast (0.001–0.003 mg/mL) and the maximum response was suppressed, similarly as with verapamil. PDE inhibitory effect of Roflumilast evaluated by recording dose-dependent (0.03–0.1 mg/mL) responses, the isoprenaline-induced inhibitory dose response curves shifted leftward similar to papaverine (PDE inhibitor). Pretreatment with Roflumilast exhibited elevated intracellular cAMP levels in tracheal strips. Findings of the experiment conclude bronchodilatory influence of Roflumilast via PDE and Ca^{++} channel inhibition. Results of current experiment offers comprehensive mechanistic background of Roflumilast in future as therapeutic bronchodilator for hyperactive bronchial airway diseases.

1. Introduction

Commonly, respiratory diseases are the consequences of overactive airway smooth muscles. Asthma is a congestive respirational malady, symptomatically recognized by dyspnea, coughing and tightness in chest, because of airflow hindrance. The universal occurrence of asthma elevating especially in youngsters. As per World Health Organization (WHO) statistics, asthma affects 5–10% of adults and 10% of children worldwide (WHO, 2007). Statistical data of global initiative for asthma suggests that bronchial asthma affects nearly 18% population globally (Loftus and Wise, 2016). Further according to a recent review report asthma is one of the most occurring noncommunicable malady among children. The total economic expenditure on asthma estimated 82 billion dollars in united states alone in 2013 (Stacy, Hao and Cheng, 2020). Therapeutic options for obstructive airway disorders principally for asthma are not considered safe and effective moreover found away from the reach of enormous patients globally. There are three broad pharmacological categories:1) drugs for Chronic management, 2) Faster and short acting drugs; and 3) biological agents (Stacy, Hao and Cheng, 2020). The existing β2 agonists have numerous unwanted effects in elderly, the corticosteroids are also unpalatable for the elderly because of ceratin serious syetemic adverse effects, theophylline belongs to one of the most important

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drugs which have interactions with numerous chemical substances and anticholinergics have been proved to have unpleasant taste and dry mouth (Donald Mackenzie Newnham, 2001) Therefore it is necessory to develop more effective and safer alternative measures which can cover all age groups safely and effectively.

Respiratory disorders encompasses numerous biological progressions, inflammatory and immunological responses, vascular confrontation, cardiac ejection volumes, dynamics of visceral organs which mainly be governed by intracellular cAMP and cGMP levels (Ghosh et al., 2009; Houslay & Adams, 2003). PDE transforms biologically to cAMP and cGMP into their inactive metabolites and known to have crucial role to guideline of innumerable functions in airway smooth muscles (Imam et al., 2018).

PDE expressions has been confirmed in numerous tissues, variation in different PDE isoenzymes expressions/action and cAMP signaling in Respiratory tract have been acknowledged (Amsallem et al., 2005). Inhibition of PDEs pharmacologically demonstrated a leading target in bronchoconstriction and asthma treatment (Imam et al., 2018). Researchers are more attentive to develop new therapeutics which can target PDE selectively (Imam et al., 2016). Type-4 isoform of PDE (PDE4), abundantly present in respiratory muscles, immune cells, heart and brain tissues (Muller et al., 1996). Earlier research are in agreement that PDE4 inhibition elevates cAMP inside the cell and stimulates various cell signaling molecules/pathways that instigate protein kinase A (PKA) and several other cAMP sensitive transcription factors (CREB/ATF-1), and down regulate expressions of a well known inflammatory mediator nuclear factor kappa-B (NF-κB) (Baldwin, 2001). Roflumilast is chemically a benzamide derivatives (Fig. 1.). Roflumilast, potently and selectively inhibit PDE4 and proposed to suppress inflammation via cAMP/PKA signaling pathways and overwhelming lipoxygenase enzymes (Erdogan et al., 2007). Hence, Considering PDEs as potential target in respiration, PDE inhibition will explore and open new doors for potential development of new applications for treatment of respiratory diseases.

By reviewing above and many more literature we came to conclude that till now Roflumilast lacks studies to evaluate its therapeutic potential in lung diseases. This particular experimental study was designed to explore the possible bronchodilatory mechanism of Roflumilast, via inhibition of PDE and Ca^{+2} channels.

2. Materials and methods

2.1. Chemicals and animals

CCh, verapamil, papaverine and isoprenaline, were procured from Sigma Aldrich St. Louis, USA, potassium chloride (Sigma Aldrich), $CaCl_2$, EDTA, glucose, $MgSO_4$, KH_2PO_4, $NaHCO_3$ and NaCl from E. Merck, Darmstadt, Germany. All chemicals used in whole experiment were of high purity.

Male/female Guinea-pigs (500–550 g) were procured and maintained at optimum laboratory conditions of relative humidity (55 ± 5%), temperature (22 ± 2 °C), and 12 h/12 h light/dark cycle at Animal house facility, College of Pharmacy, Prince Sattam bin Abdulaziz University, Saudi Arabia. Animals provided with Normal feed and water *ad libitum*. All Experimental procedures were carried out under standard rulings of Animal ethics committee (IRB No.: BERC-004-12-19).

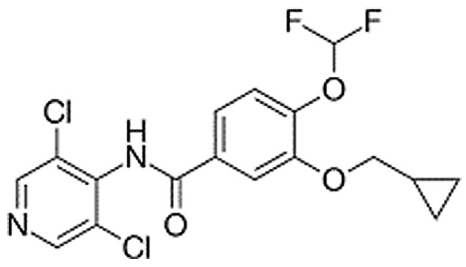

Fig. 1. Chemical structure of Roflumilast (https://pubchem.ncbi.nlm.nih.gov/compound/Roflumilast).

2.2. In-vitro testing of Roflumilast on spiral strips of tracheal chain

Guinea pigs were sacrificed by hanging drop method and trachea isolated and instantly provided with fresh Kreb's solution. The tracheal preparation of 2–3 mm wide taken and each ring opened by cutting vertically the anteriorly reverse to smooth muscle layer, tracheal chain spiral strips were made using a central smooth muscle of the middle portions of cartilaginous portions on the edges. Tracheal chain spiral preparations mounted in 20 mL organ tube containing physiological salt solution (Kreb's) at 37 °C provided with carbogen (95:5) of Oxygen and Carbon dioxide. The ingredients of physiological solution were as follows (mM): NaCl 118.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, KCl 4.7, KH_2PO_4 1.3, $MgSO_4$ 1.2 and glucose 11.7 (pH 7.4). 1 g of tension applied to the mounted tissue preparation constantly throughout whole experiment. Tissue preparation was stabilized for at least 1 h prior to exposure of test substances. CCh and K^+ added to organ tube for stabilization of particular preparations, till achievement of constant responses of agonists. Later persistent contractile responses were recorded and relaxant influence of test chemicals was evaluated in a cumulative fashion to achieve concentration-dependent curves of inhibition. Increased K^+ (**80** mM) cause contraction in smooth muscle via stimulating voltage-sensitive L-type Ca^{++} channels, invasion of extracellular Ca^{++} inside the cell triggers contraction the drugs which inhibit K^+-induced contraction inhibits Ca^{++} influx ultimately (Godfraind et al., 1986; Bashir et al., 2011). The PDE inhibitory potential was examined via recording inhibitory responses of isoprenaline against CCh-leaded contractions with or without test substance, as stated earlier (Gilani et al., 2005). Isometric contraction recorded utilizing isolated EmkaBath (France) and IOX software.

2.2.1. Effect on Ca^{+2} channel inhibition

For confirmation of Ca^{+2} channel inhibition of the drug under study, tissue was mounted in tissue bath and equilibrated with Kreb's solution which was later changed to Ca^{+2}-restricted Kreb's containing EDTA (0.1 mM) for 30 min to eliminate Ca^{+2} from the tissues. The physiological salt solution again replaced with K^+-saturated and Ca^{+2}-free Kreb's, (KCl 50, NaCl 50.58, $MgSO_4$ 3.10, $NaHCO_3$ 23.8, KH_2PO_4 1.26, glucose 11.1 and EDTA 0.1 {mM}). After incubation of 60 mins, Normal control responses of Ca^{+2} recorded. After receiving constant responses of Ca^{+2} (usually after two cycles), the tissue was soaked with test substance for 60 min. Ca^{+2} induced contraction responses recorded again at increasing concentration of the test.

2.2.2. PDE enzyme inhibition

Inhibition of PDE was examined indirectly from isoprenaline induced inhibitory concentration responses against carbachol (CCh)-leaded contraction responses with or without test sample (Gilani et al., 2005).

2.2.3. Effect Roflumilast on intracellular cAMP level

Isolated trachea from animal spontaneously freezed in liquid nitrogen, finely powdered by homogenizing with 10 vol (0.1 M) HCl under liquid nitrogen. Resultant centrifuged at 600xg at normal temperature for 10 min, further diluted using (0.1 M) HCl and put away at − 80 °C till assayed. The impact of roflumilast on intracellular cAMP level was assayed using immunoassay kits,

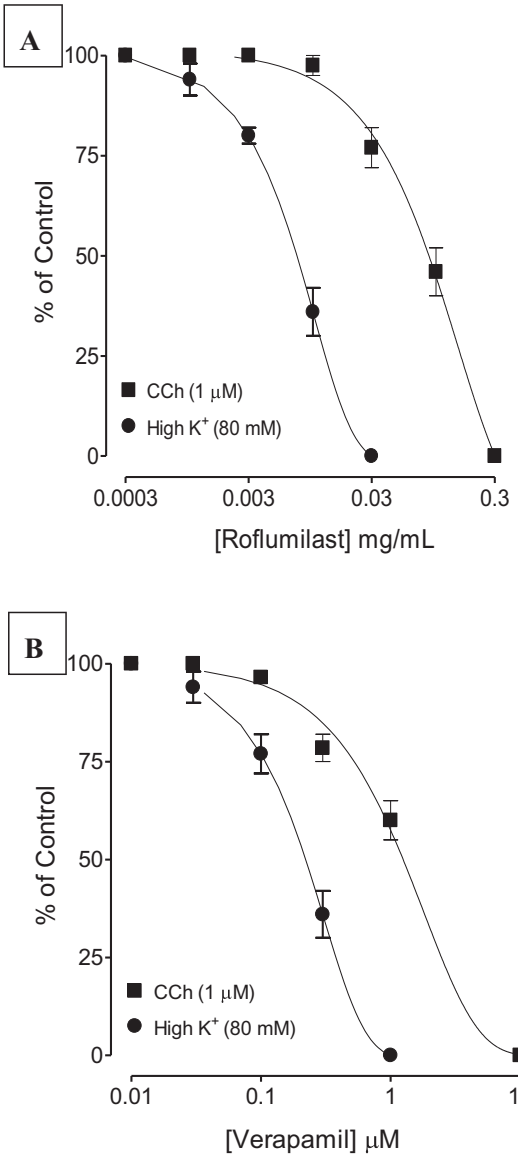

Fig. 2. Concentration-response curves showing comparison of **(A)** Roflumilast and **(B)** verapamil for the inhibitory effect against carbachol (CCh, 1 μM) and high K^+ (80 mM)-induced contractions in isolated guinea-pig tracheal preparations. Values shown are mean ± SEM, n = 4–5.

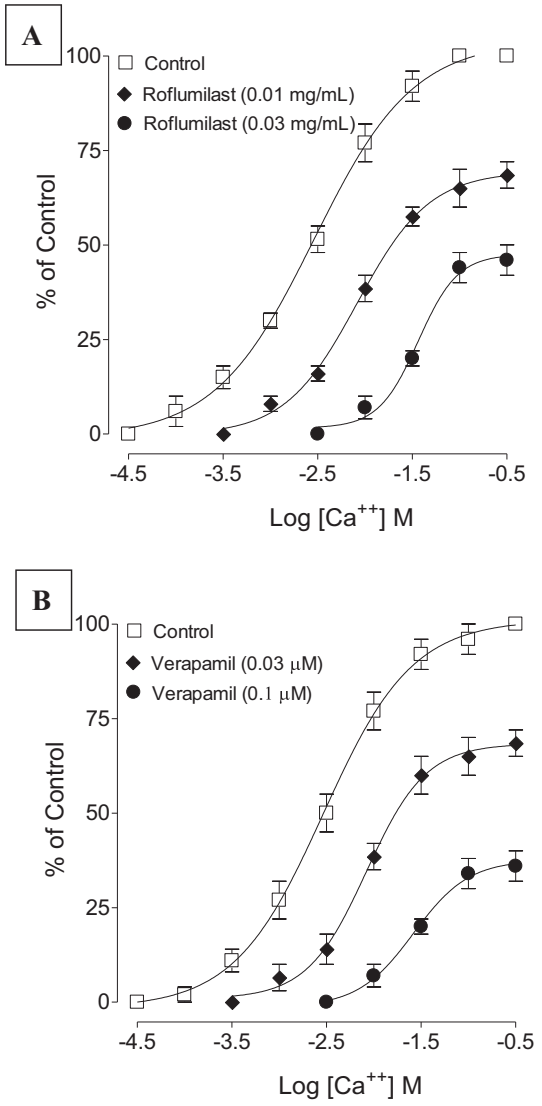

Fig. 3. Concentration-response curves of Ca^{++} in the absence and presence of the increasing concentrations of **(A)** Roflumilast and **(B)** verapamil in isolated guinea-pig tracheal preparations. Values shown are mean ± SEM, n = 4–5.

strictly adhering to manufacturer procedures (Sigma-Aldrich, USA) and reported as picomole per mL.

2.3. Statistical analysis

Data presented as mean ± SEM, and effective concentration 50% (EC_{50}) with 95% confidence intervals (CI). Statistical analysis done via GraphPad prism software (GraphPAD, San Diego, CA, USA) ANOVA followed by Dunnett test to compare the results. $p < 0.05$ is deliberated statistically significant.

3. Results

3.1. Effect of roflumilast on isolated trachea

Findings of the research clearly depicted that roflumilast inhibited contractions which were brought by CCh exposure (1 μM) and K^+ (80 mM), and found more powerful to suppress K^+ leaded contractions as compared to CCh with respect to EC_{50} values of 0.02 mg/mL (0.014–0.026, 95% CI, n = 4) and 0.1 mg/mL (0.08–0.14, n = 5) Fig. 2**A**. There were no significant differences found with that of Verapamil which also showed analogous inhibition in CCh and high K^+-leaded contractions with EC_{50} values of 0.14 μM (0.11–0.19, n = 4) and 1.4 μM (1.22–1.86, n = 4)] Fig. 2**B**.

3.2. Effect of roflumilast on Ca^{++}-Curves

Findings revealed possible interaction with Ca^{++} channels, which was evident by a rightward shift in the Ca^{++} curves after pretreatment with Roflumilast (0.01–0.03 mg/mL) (Fig. 3A) when compared, results were found comparable to that of verapamil (Fig. 3B).

3.3. Effect of roflumilast on isoprenaline-Curves

Pretretment with Roflumilast (0.03 and 0.1 mg/mL), the isoprenaline-leaded inhibition shifted leftward (Fig. 4A), Which demonstrated potentiation of relaxant effect of isoprenaline.

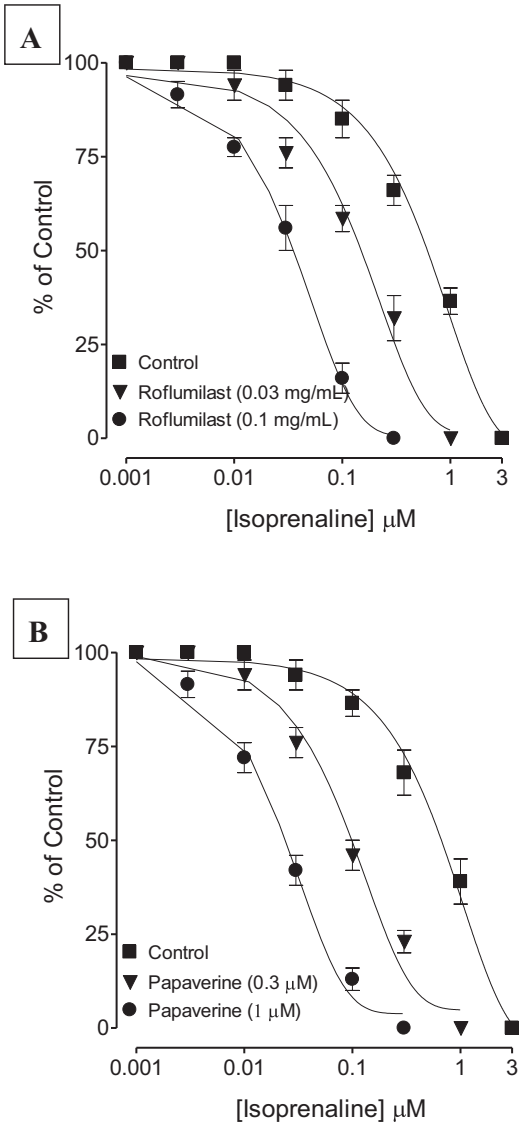

Fig. 4. Inhibitory concentration–response curves of isoprenaline against carbachol (CCh)-induced contractions in the absence and presence of different concentrations of **(A)** Roflumilast and **(B)** papaverine, in isolated guinea-pig tracheal preparations. Values shown are mean ± SEM, n = 4–5.

Papaverine (0.3–1 μM) administration leaded to leftward shift similarly Fig. 4B.

3.4. Effect of roflumilast on cAMP levels

cAMP assay results suggest that intracellular cAMP in untreated tissues exhibited 90.31 ± 0.95 pmol of cAMP/mL, while the groups exposed to roflumilast 0.03 and 0.1 mg/mL against CCh contractile responses, had 97.78 ± 1.82 ($p < 0.01$) and 99.74 ± 0.93 ($p < 0.01$) pmol/mL, respectively (Fig. 5A). Exposure of papaverine caused similar elevation in cAMP levels, against CCh treated tissues (87.99 ± 1.54 pmol/mL), whereas treatment with 0.3 and 1 μM of papaverine against CCh exposed tissues, cAMP levels elevated upto 98.00 ± 2.37 pmol/mL ($p < 0.01$) and 103.00 ± 0.82 ($p < 0.01$) pmol/mL. respectively (Fig. 5B).

4. Discussion

Earlier the researchers have reported wide therapeutic potential of Roflumilast and showed its effectiveness in several inflammatory conditions of heart and kidneys (Ansari et al., 2019a and b). Our experimental in-vitro test results demonstrate that Roflumilast, which act by inhibiting PDE-4, inhibited CCh and high K^+ tempted contractions and found particularly more effective to inhibit high K^+ leaded contractions as compared to CCh, which canuniquely be achieved by CCBs (Godfraind et al., 1986; Gilani et al., 2005). Our findings further supported, as verapamil (Fleckenstein, 1977) found to inhibit K^+-induced contractions. Further CCBs proved their usefulness in bronchoconstriction (Ahmed, 1992; Twiss et al., 2002). Remarkably, above claim was further confirmed when tracheal preparations pretreated with Roflumilast and the Ca^{+2} CRCs shifted rightward, similarly as achieved on verapamil exposure (Fleckenstein, 1977).

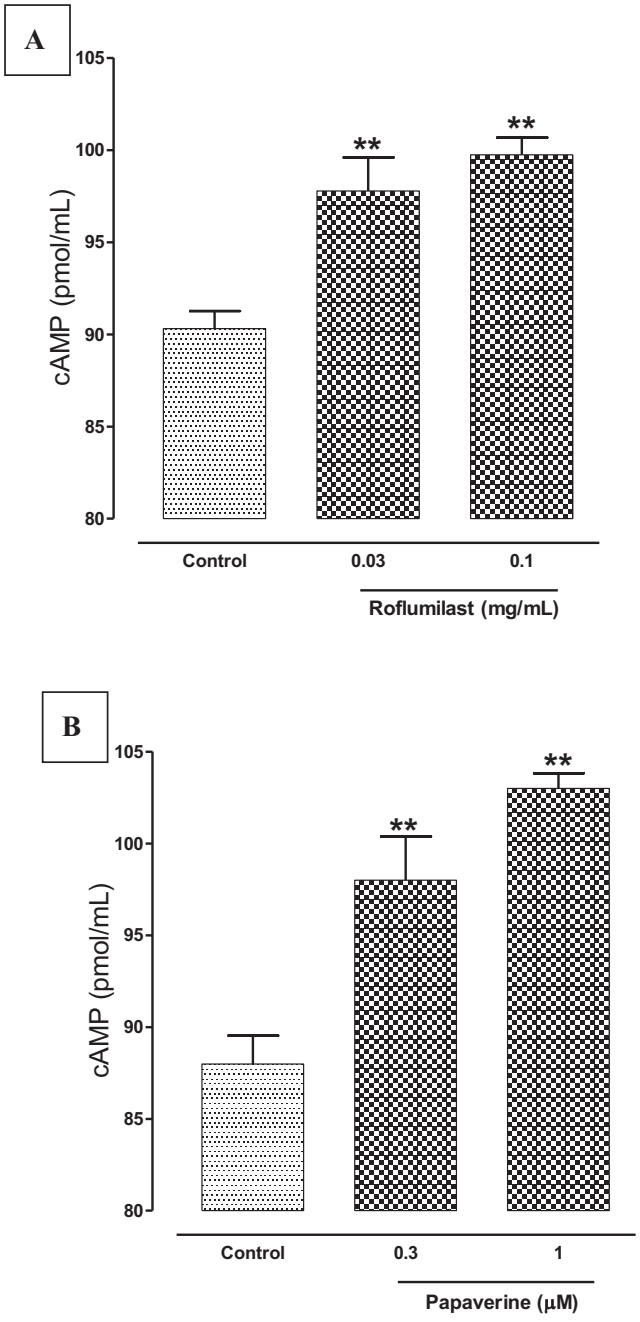

Fig. 5. Results showing effect of **(A)** Roflumilast and **(B)** papaverine, on the cyclic nucleotide (cAMP) content in isolated guinea-pig tracheal preparations. Values shown are mean ± SEM, n = 4–5.

Researchers claimed extensive involvement of PDE in hyperactive airway disease, here PDE-inhibitory activity of Roflumilast studied on isolated tracheal chain strips isolated form guinea-pig. PDE inhibitory potential evaluated via treatment of tracheal spiral strips first with increasing concentrations of Roflumilast and isoprenaline-leaded inhibitory responses were recorded against CCh. Roflumilast found to potentiate the isoprenaline-leaded relaxant effect, analogous to papaverine (Sato et al., 2006). PDE biologically transform cAMP into its metabolites and embarrassment of PDE can accumulate cAMP intracellularly (Harris et al., 1989). An elevation in cAMP level intracellularly ultimately stimulates protein kinase A enzyme (Brown et al., 2012). For further confirmation, we measured cAMP levels in tracheal tissue in the control group and compared it with Roflumilast and papaverine pretreated tracheal tissue. This finding along with concentration-dependent elevation in intracellular cAMP in CCh pre-exposed tissues further confirmed that PDE-inhibition is one of the additional mechanism (s) observed in its bronchodilatory effect. The PDE inhibitors increase cAMP levels intracellularly and are considered smooth muscle relaxants (Saegusa et al., 2003; Kaneda et al., 2005) and cardiac stimulant (Korth, 1978). Thus, inhibition of PDE and Ca^{+2} channels confirmly responsible for the observed effect of Roflumilast in hyperactive respiratory disorders, such as asthma. The PDE inhibition and its usefulness in asthma is very well studied (Barnes, 2006). Excitingly, Ca^{+2} antagonists have been confirmed to play significant role in bronchoconstriction (Twiss et al., 2002) and exhibit cardio-suppressant potentials (Billman, 1992). Thus, inhibition of Ca^{+2} channels with PDE by Roflumilast can counterbalance tachycardia, which is considered as major limited factor of PDE inhibition.

In conclusion, findings suggest that Roflumilast offers bronchodilator activities via dual mechanisms, PDE and Ca^{+2} channel inhibitions, which prove it as a potential candidate in hyperactive airways disorders.

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