ERJ Express. Published on August 13, 2009 as doi: 10.1183/09031936.00034009

β_2-agonists block tussive responses in guinea pigs via an atypical cAMP-dependent pathway

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Total word count: 2413 words

Running head: Anti-tussive activity of β_2-agonists

Abstract

β_2-adrenocpetor agonists are the most effective bronchodilators currently available and are used for symptom management in asthmatics. However, whether β_2-agonists are also anti-tussive is controversial. Identifying an anti-tussive role for β-agonists and dissecting the possible mechanism of action may help explain the inconsistencies in the clinical literature and lead to the development of novel therapeutics.

The aim of this study was to determine whether β_2-agonists attenuate the tussive response in guinea pig and human models and, if so, to identify the mechanism(s) involved.

A β_2-agonist, terbutaline, dose-dependently inhibited the cough response to tussive agents in conscious guinea pigs. Terbutaline and another β_2-agonist fenoterol blocked sensory nerve activation *in vitro.* Using these mechanistic models it was established that β_2-agonists suppress the tussive response via a non-classical, cAMP-dependent pathway that involves the activation of PKG and, subsequently, the opening of the large-conductance calcium-activated potassium (BKCa) channels.

In conclusion, β_2-adrenoceptor agonists are anti-tussive and this property is due to a direct inhibition of sensory nerve activation. These findings may help to explain the confusion that exists in the clinical literature and could be exploited to identify novel therapies to treat cough which presents as a significant unmet medical need.

Keywords: airway sensory nerves, β_2-adrenoceptor agonist, cough, cyclic AMP, large conductance calcium-activated potassium channels (BKCa channels), protein kinase G

Introduction

β_2-adrenoceptor agonists (β_2-agonists) are currently one of the most effective bronchodilators available; they have been used to alleviate bronchoconstriction in respiratory diseases such as asthma for many decades [1]. However, despite their prolific use, there is still controversy as to whether they also possess anti-tussive properties. Clinical studies conducted so far have not managed to clearly establish whether β_2-agonists attenuate cough. Indeed, while some clinical studies in normal volunteers [2] and in chronic cough associated with allergic [3] or obstructive conditions [4-7] have found a beneficial effect of β_2-agonists, other trials have not found efficacy [8, 9]. This confusion may exist, in part, due to the subjective nature of symptom scoring in comparison with objective cough monitoring which has only recently been made available and the fact that few double blind, randomised placebo controlled studies have been performed with cough as a primary end point. This is important given patients (especially those with iodiopathic cough) find it very difficult to make an accurate assessment of their cough. Furthermore, it is commonly believed that if β_2-agonists do, indeed, suppress cough then bronchodilation is the likely mechanism of action [7, 8].. Currently the dose regimen/protocol for β_2-agonists in the clinic is routinely based around their relaxant properties and not geared to their anti-tussive activities (which may require higher doses) and we propose that this may be why a dominant anti-tussive property has not hitherto been discovered.

Cough is a protective reflex in healthy individuals [10]. However, chronic cough is associated with many inflammatory airway diseases, and is a very common and troublesome symptom in particular in asthma or in chronic obstructive pulmonary diseases (COPD) [11, 12]. Cough is the most common respiratory complaint for which medical attention is sought (for review: [13]) However, currently treatments for cough are very poor; most “over-the-

counter" remedies are not effective and prescribed medications tend to work centrally thereby rendering them inappropriate for most sufferers as in many cases they cause sedation. Clearly, understanding the mechanism behind any anti-tussive action of β_2-agonists could lead to new therapies that may impact on this unmet clinical need.

We hypothesised that β_2-agonists possess anti-tussive properties through directly inhibiting sensory nerve activity, independently from bronchodilation. Confusion exists in the clinical literature surrounding this property of β_2-agonists and this may be due to the fact that the signal transduction mechanisms that elicit bronchodilation are different to those that induce anti-tussive activity. Accordingly, we report here the results of studies in which we have determined whether a β_2-agonist attenuates the cough response induced by clinically-relevant tussive stimuli *in vivo.* We have also examined the effect of β_2-agonists on guinea pig and human sensory nerve activity *in vitro*. This isolated preparation circumvents complicating issues such as bronchodilation and allows the signalling pathways that regulate cough due to changes in sensory nerve activity to be probed. These experiments are not aimed primarily at making a case for using β_2-agonists as anti-tussive therapies but to uncover an alternative signalling pathway from which new targets could be uncovered.

Material and methods

Animals

Male Dunkin-Hartley outbred guinea pigs (300-500g) were purchased (Harlan, Bicester, Oxon, UK). Experiments were performed in accordance with the UK Home Office guidelines for welfare based on the Animals (Scientific Procedures) act 1986.

Materials

1-(2'hydroxy-5'-trifluoromethylphenyl)trifluoro-methyl-2-[^{3}H]benzimidazolone (NS1619) was from Research Biochemicals Inc. (St Albans, UK). All other chemicals were from Sigma Aldrich (Poole, UK).

Measurement of cough in vivo in the conscious guinea pig

Measurement of cough in conscious guinea pigs was previously described [14-17]. Terbutaline (0.05-3mg/kg i.p., 1ml/kg) or vehicle (0.5% methylcellulose / 0.2% Tween 80 in saline) was administered 30 min prior to exposure to capsaicin (10^{-4}M, 5 min) or citric acid (0.3M, 10 min) and the number of coughs over a 10 min period was recorded.

Measurement of sensory nerve depolarisation of isolated vagus nerve preparations in vitro

To demonstrate an inhibitory action of β_2-agonists on sensory nerve activation we used the fully characterized isolated vagal nerve preparation as described in previous publications (14, 17). Tussive agents such as capsaicin and low pH solutions are known vagal sensory nerve stimulants and as such isolated guinea pig and human vagus nerve preparations have been shown to elicit nerve depolarisation responses to these stimulants (14, 17). Furthermore, these agents are also known tussigenic agents in human and animal studies (16, 18). These data suggest that the isolated vagus nerve is an ideal preparation for conducting comprehensive pharmacological assessments of agents that may activate or inhibit sensory nerve function and

thus the cough reflex. Previous data also suggest that the information generated is broadly predictive of data generated in single afferent nerve fibre recording studies further validating the use of the isolated vagus preparation as an *in vitro* indicator of the cough reflex (15).

Depolarisation was induced by superfusion of isolated nerves with capsaicin (10^{-6}M in guinea-pig and 10^{-5}M in human tissue) or low pH solution (pH 5) for 2min. The nerves were then washed until the responses had returned to baseline. Following two reproducible depolarisations, the nerves were superfused with terbutaline ($3x10^{-6}$ to $3x10^{-4}$M) or vehicle for 10min. The responses to capsaicin or citric acid solution were then measured in the presence of terbutaline or its vehicle (0.1% DMSO). After washing, recovery was observed by superfusing the nerves again with the depolarising agents for 2min. In some experiments, vagus nerves were pre-incubated (10 min) with antagonists, enzyme inhibitors or channels blockers before superfusion with terbutaline.

Human vagus nerves were obtained from donor tissue for heart/lung transplantation with relevant approvals obtained from the Royal Brompton and Harefield Trust Ethics Committee.

Measurement of cyclic adenosine-3',5'-monophosphate (cAMP) levels in isolated guinea pig vagus nerve preparations in vitro

Desheathed vagus nerves were incubated in terbutaline ($3x10^{-4}$M) or vehicle (0-10min, 37°C), then frozen in liquid nitrogen and ground to a fine powder. Frozen tissues were weighed, homogenised in 10 volumes of 0.1M HCl and centrifuged at 600*g*. cAMP levels were determined in the supernatants using a commercial enzyme immunoassay kit (cAMP EIA Kit, Biomol Research Laboratories Inc., Plymouth Meeting, PA, USA) according to the manufacturer's instructions. cAMP levels were normalised to the frozen tissue weight. For

each animal, one vagus nerve was treated with terbutaline while the second was used as a paired time-matched control.

Expression of results and statistical analysis

Data are expressed as the mean ± S.E.M. of n independent observations. Nerve depolarisation is expressed as mV before and after drug addition and presented as a percentage change. cAMP levels are expressed in pmol/mg of frozen tissue. Parametric data were analysed by a two-tailed Student's t test when comparing two groups, or by a one-way ANOVA followed by a Student-Newman-Keuls multiple comparison test, or followed by a Dunnett's multiple comparison test when comparing several treatment groups to the same control. Non-parametric data were analysed by a Kruskal-Wallis test followed by a Dunn's multiple comparison test. All data were analysed using GraphPad PRISM (Graphpad Software, San Diego, CA, USA). A p value less than 0.05 was considered statistically significant.

Results

Terbutaline inhibits cough in the conscious guinea pig

Capsaicin (10^{-4}M) induced cough in the conscious guinea pig and this response was dose-dependently inhibited by terbutaline, with a maximal inhibition of 84% at 0.3mg/kg ($p<0.05$, **Figure 1A**). Similarly, terbutaline dose-dependently inhibited cough elicited by a citric acid (low pH) solution (0.3M), with a maximal inhibition of 85% at 1mg/kg ($p<0.05$, **Figure 1B**). Vehicle had no effect on cough induced either by capsaicin or by citric acid.

β_2-adrenoceptor agonists inhibit depolarisation of the isolated vagus nerve

Capsaicin and citric acid solution elicited reproducible depolarisation of the guinea pig vagus nerve of 0.28 ± 0.05 and 0.18 ± 0.03mV, respectively. Pre-treatment of preparations with terbutaline inhibited nerve depolarisation induced by both capsaicin (10^{-6}M) and low pH solution (pH5), in a concentration-dependent manner and with a maximal inhibition at $3x10^{-4}$M ($p<0.001$ for both, **Figures 2A and 2B**). Pre-treatment of nerves with fenoterol (10^{-4}M) another β_2-adrenoceptor agonist, inhibited capsaicin-induced depolarisation of the guinea pig vagus nerve by 84% **(Figure 2C)**. The effect of both terbutaline and fenoterol (10^{-4} M) on nerve depolarisation was abolished by the selective β_2-adrenoceptor antagonist ICI118551 (1μM, $p<0.001$), showing involvement of the β_2-adrenoceptor **(Figure 2C)**. Finally, the inhibitory effect of β_2-adrenoceptor agonists was also seen in the human vagus, since fenoterol (10^{-4}M) inhibited the depolarisation of isolated human vagus nerve preparations induced by capsaicin **(Figure 2D)**.

All the vehicles tested did not have any effect on vagus nerve depolarisation. Further experiments on depolarisation of guinea pig isolated vagus nerve preparations were then performed with capsaicin using terbutaline at a sub-maximal concentration of 10^{-4}M.

Increases in cAMP inhibit nerve depolarisation

Capsaicin-induced depolarisation of the guinea pig vagus was inhibited in a concentration-dependent manner by pre-treatment with 8-Br-cAMP (a cAMP analogue), rolipram (a PDE4 inhibitor), or forskolin (an activator of adenylyl cyclase), with maximal inhibition at 10^{-4}M ($p<0.05$ for all, **Figure 3A**). 1,9-dideoxyforskolin, an inactive analogue of forskolin, was inactive at all concentrations tested **(Figure 3A)**. Terbutaline ($3x10^{-4}$M) produced a transient increase in cAMP levels in the guinea pig vagus nerve relative to time-matched controls, with a maximal 2-fold increase after 20sec of treatment ($p<0.05$, **Figure 3B**).

Involvement of adenylyl cyclase and protein kinase G (PKG), but not protein kinase A (PKA)

Terbutaline-induced inhibition of capsaicin-elicited depolarisation of the guinea pig vagus nerve was abolished by the adenylyl cyclase inhibitor SQ 22,536 ($3x10^{-4}$M, $p<0.05$) (**Figure 4A**) but unaffected by KT5720, a selective PKA inhibitor (**Figure 4B)**. In contrast, the inhibitory effect of terbutaline was blocked by the selective inhibitor of PKG KT 5823 (87% inhibition, $p<0.05$) (**Figure 4B**).

Involvement of large-conductance calcium-activated potassium (BKCa) channels

Involvement of the different potassium channels was investigated by use of the following inhibitors: paxilline (10^{-6}M) to block large-conductance calcium-activated potassium channels (BKCa channels); clotrimazole (10^{-5}M) to block intermediate conductance calcium-activated potassium channels (IKCa channels); apamin (10^{-6}M) to block low-conductance calcium-activated potassium channels (SKCa channels) and glibenclamide (10^{-5}M) to block ATP-activated potassium channels (K^{+}_{ATP} channels). Of these channel blocking drugs tested only

paxilline reversed the inhibitory effect of terbutaline on capsaicin-induced depolarisation ($p<0.001$, **Figure 5A**).

In separate experiments, depolarisation of the guinea pig vagus nerve was also induced by the endogenous activators, prostaglandin E_2 (10^{-5}M) and bradykinin (10^{-5}M). These stimuli elicited nerve depolarisation of 0.12 ± 0.02 and 0.06 ± 0.01 mV respectively. Pre-treatment of the preparation with terbutaline inhibited both PGE_2- and bradykinin-induced nerve depolarisations by 95% and this effect was prevented by paxilline ($p<0.001$, **Figure 5B**).

NS1619 has previously been shown to inhibit both the firing of single fibres in airway sensory nerves *in vitro* and guinea pig cough *in vivo*, through activation of BKCa channels [15]. NS1619 ($3x10^{-5}$M) was therefore tested on the guinea pig vagus nerve to validate the concept of inhibition of vagal activity through opening of BKCa channels and was found to inhibit nerve depolarisation by 96% ($p<0.001$, **Figure 5C**). Finally, paxilline (10^{-6}M) was also able to reverse the inhibitory effect of fenoterol (10^{-4}M) on capsaicin-induced depolarisation of the guinea pig vagus by 86% ($p<0.05$, **Figure 6A**), and reversal of the fenoterol effect by paxilline was also demonstrated in a isolated human vagus preparation (**Figure 6B**).

Discussion

In this study, we demonstrate anti-tussive activity of β_2-agonists in a pre-clinical model of cough in the guinea pig *in vivo* and report that this class of drugs directly inhibits sensory nerve activity *in vitro*. Moreover, our findings were reproduced in the human vagus implying that β_2-agonists may possess anti-tussive activity in man. Mechanistically, β_2-agonists appear to inhibit depolarisation of the guinea pig vagus nerve by stimulating adenylyl cyclase, which leads to cAMP accumulation, activation of PKG and activation of BKCa channels which presumably inhibits sensory nerve activation and therefore the cough reflex.

In the past, the anti-tussive activity of β_2-agonists, when found, was attributed to relaxation of the airway smooth muscle [9]. However, to date clinical studies conducted to investigate the impact of β_2-agonists on the tussive response have led to contradictory results [1-5, 7-9]. Presumably due to the subjective nature of reporting in clinical trials prior to the advent of objective cough monitoring. Moreover, to our knowledge the precise mechanism of action for this potential antitussive effect has not been studied. Herein, we demonstrate that a β_2-agonist attenuates the cough response induced by capsaicin and citric acid. These tussive agents are commonly used in humans [19] and in pre-clinical *in vivo* models, and this inhibitory effect of a β_2-agonist is in accordance with previous results observed in a similar model [20, 21].

To circumvent the potentially confounding bronchodilator properties of β_2-agonists and to aid in understanding how the β_2-agonist is inhibiting cough, we used an isolated vagus preparation to study sensory nerve activity. This preparation has previously been characterised and shown to respond to a range of pre-clinical and clinical tussive agents. Moreover, this preparation provides the opportunity to conduct a comprehensive pharmacological assessment, to assess the direct action of drugs without pharmacokinetics

issues, and to dissect the signalling pathways involved. However, data obtained by using this model should be interpreted with some caution as drugs are applied on the axon of the isolated vagus nerve preparation and not the nerve terminal. The depolarisation recorded is therefore the summation of the changes in membrane potentials of all the axon due to activation of receptors on the axon itself, and these receptors may also not reflect the receptors and signalling pathways at the peripheral endings [14, 17]. However, responses in guinea pig and human tissues are comparable, and results appear to translate to *in vivo* models, thus suggesting that such preparations are amenable for studying the mechanisms involved in sensory nerve modulation [14, 17, 22].

Using this system we demonstrated that agonism of the β_2 receptor attenuated capsaicin- and citric acid-induced sensory nerve activation. Using a pharmacological approach we further established that both terbutaline and fenoterol were acting through the β_2 receptor. Moreover, by reproducing the effects in human vagal sensory tissue we demonstrated that this phenomenon may translate into man. Finally, we demonstrated that a β_2-agonist inhibits sensory nerve depolarisation elicited by PGE_2 or bradykinin, which are endogenous tussive mediators known to modulate sensory nerves in man [23]. This suggests that β_2-agonists could be effective against a range of exogenous and endogenous tussive stimuli.

Terbutaline induced an elevation of cAMP in the vagus nerve. Moreover, an inhibitor of adenylyl cyclase, SQ 22,536, abolished terbutaline-induced inhibition of nerve depolarisation whereas the cAMP analogue, 8-Br-cAMP, and other agents known to raise cAMP, such as forskolin or a PDE4 inhibitor, mimicked the effect of terbutaline. This finding is consistent with activation of the classical β_2 receptor signalling pathway that involves coupling of the β_2-receptor to adenylyl cyclase (for review: [24]). Our results therefore

demonstrate a central role for cAMP in the signalling pathway activated by β_2-agonists that leads to the inhibition of sensory nerve activity.

The current dogma suggests that β_2-agonists relax airway smooth muscle via the cAMP-dependent activation of PKA [24, 25]. In the present study, however, terbutaline-induced inhibition of guinea pig vagus nerve depolarisation was unaffected by KT5720, an inhibitor of PKA, whereas KT5823, an inhibitor of PKG, abolished the effect of terbutaline. As the level of cyclic guanosine-3',5'-monophosphate (cGMP) was not elevated in the vagus (data not shown), these data suggest that cAMP cross-activates PKG. This phenomenon has been observed previously [26-28] leading to the suggestion that β_2 receptors can activate PKA-independent mechanisms to elicit functional responses [29-33]. If the bronchodilator and anti-tussive activity of β_2-agonists is mediated by different signalling mechanisms it could explain some of the discrepancies in the clinical literature (given that anti-tussive effects of β-agonists are not reported in every study where this endpoint has been evaluated) as the dose regimen/protocol is routinely based around their relaxant properties and perhaps are not geared to their anti-tussive activities.

To determine the potential direct or indirect target of PKG that could lead to inhibition of depolarisation in the vagus, we used a range of ion channel blocking drugs. Our results clearly demonstrate a role for the large-conductance calcium-activated potassium (BKCa) channels since paxilline, a blocker of these channels, markedly inhibited the terbutaline response. Moreover, NS1619, which activates BKCa mimicked the effect of terbutaline. These results are in accordance with data previously generated by us and others [34-37]. Significantly, paxilline also reversed fenoterol-induced inhibition of capsaicin-induced depolarisation in the human vagus nerve indicating that the signalling mechanism established in the guinea pig may be extrapolated to humans.

In conclusion, our data show that β_2-adrenenoceptor receptor agonists are anti-tussive and that this property is due to a direct inhibition of sensory nerve activation and is not due to the signalling pathways thought to be involved in bronchodilation. We suggest that these findings could explain the inconsistencies in the clinical literature regarding the variable anti-tussive activity of β_2-agonists. Currently the dosing regimen/protocol for β_2-agonists in the clinic is routinely based around their relaxant properties and is not geared to their anti-tussive activities (which, for example, may require higher doses) and we propose that this is why a dominant anti-tussive property has not been discovered in patients. In animals studies we are not dose-limited in this regard and so this may reveal clear anti-tussive activity which is often missed in clinical studies. The message from this study is not specifically to promote β_2-agonists as anti-tussive therapies but to uncover an alternative signalling pathway to be exploited in order to identify appropriate therapies to treat the unmet medical need of cough.

Acknowledgments

The human tissue work was funded by an Experimental Medicine grant (G0502019) from the Medical Research Council, UK. Véronique Freund-Michel is the recipient of a European Respiratory Society Fellowship (Number 154).

The human tissue experiments in this manuscript were undertaken with the support of the NIHR Biomedical Research Unit in Advanced Lung Disease at the Royal Brompton and Harefield NHS Foundation Trust and Imperial College London and partly funded by the NIHR Biomedical Research Unit funding scheme.

The authors thank Dr Natascia Crispino and Dr Hema Patel for their assistance with some of the *in vitro* experiments included in this manuscript.

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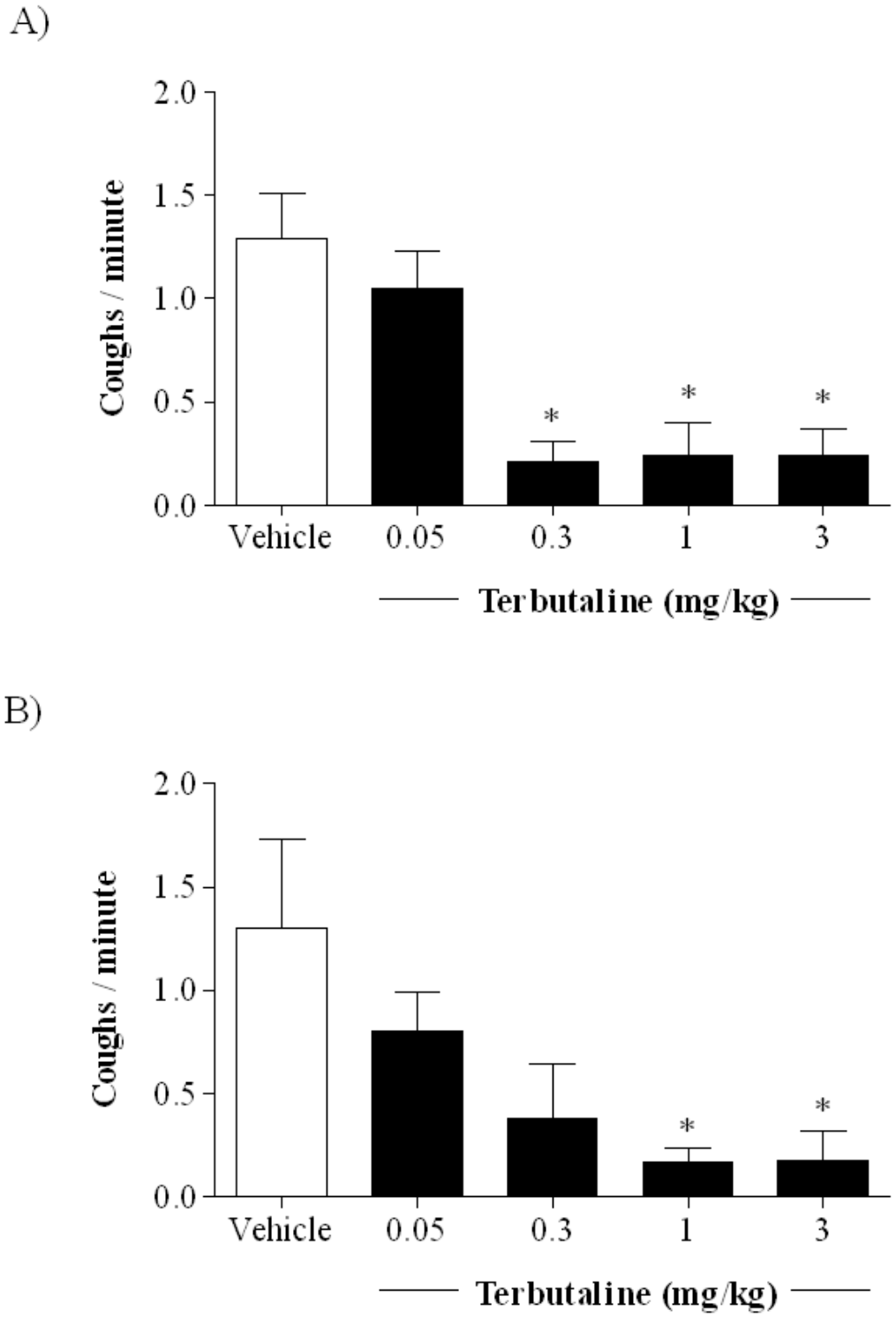
Figure 1


A)
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Terbutaline (mg/kg)

Figure 2

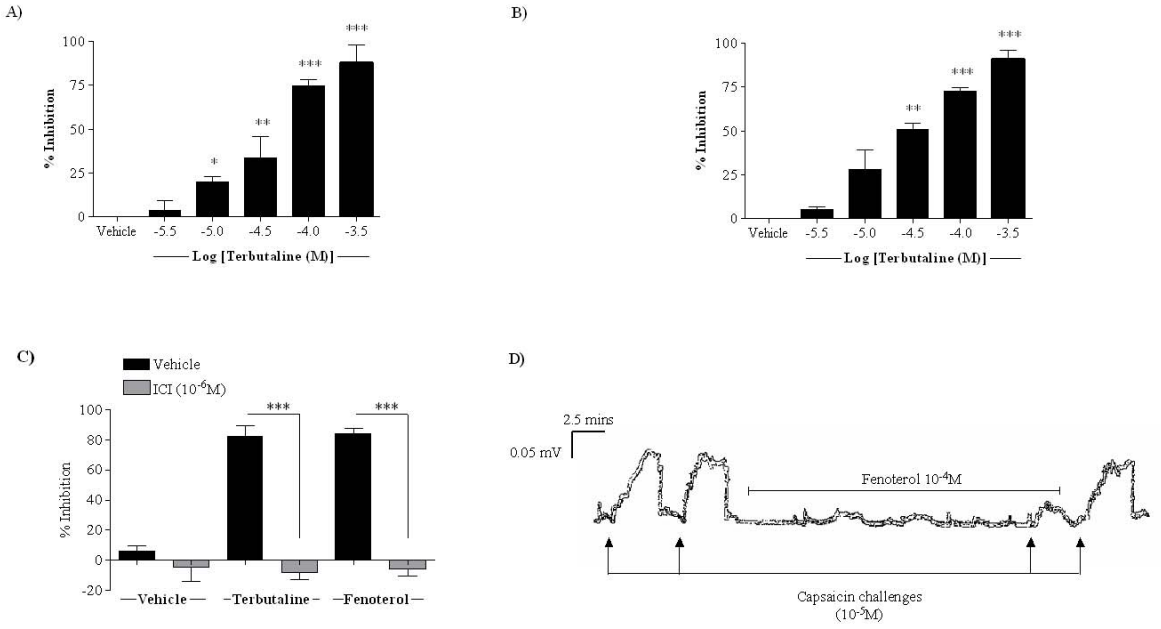
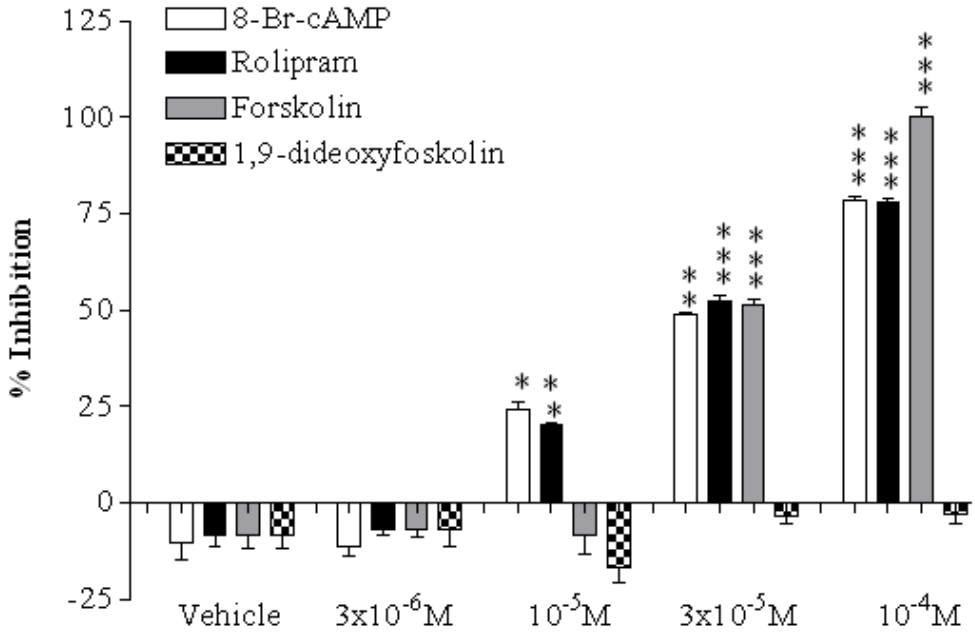


Figure 3

A)


8-Br-cAMP
Rolipram
Forskolin
1,9-dideoxyfoskolin
% Inhibition
125
100
75
50
25
0
-25
Vehicle
3x10-6M
10-5M
3x10-5M
10-4M

B)

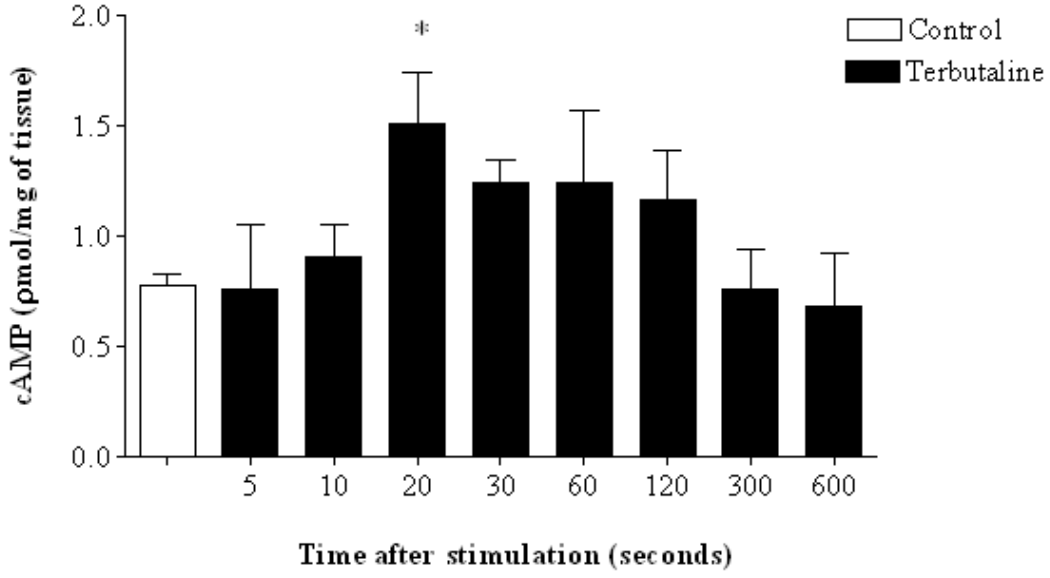
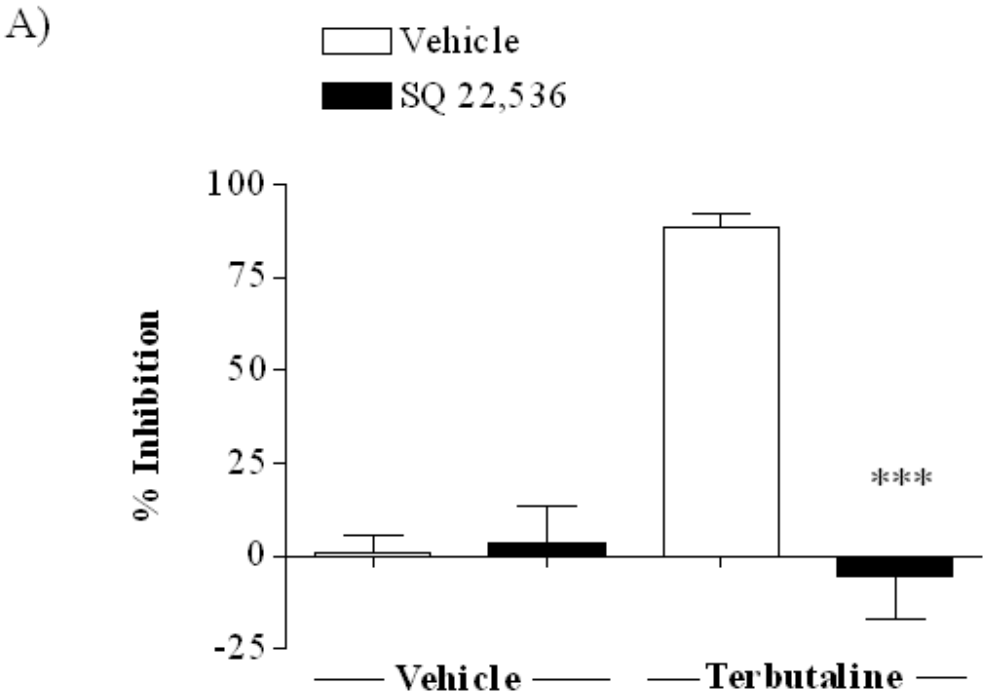

Control
Terbutaline
cAMP (ρmol/mg of tissue)
2.0
1.5
1.0
0.5
0.0
5
10
20
30
60
120
300
600
Time after stimulation (seconds)

Figure 4



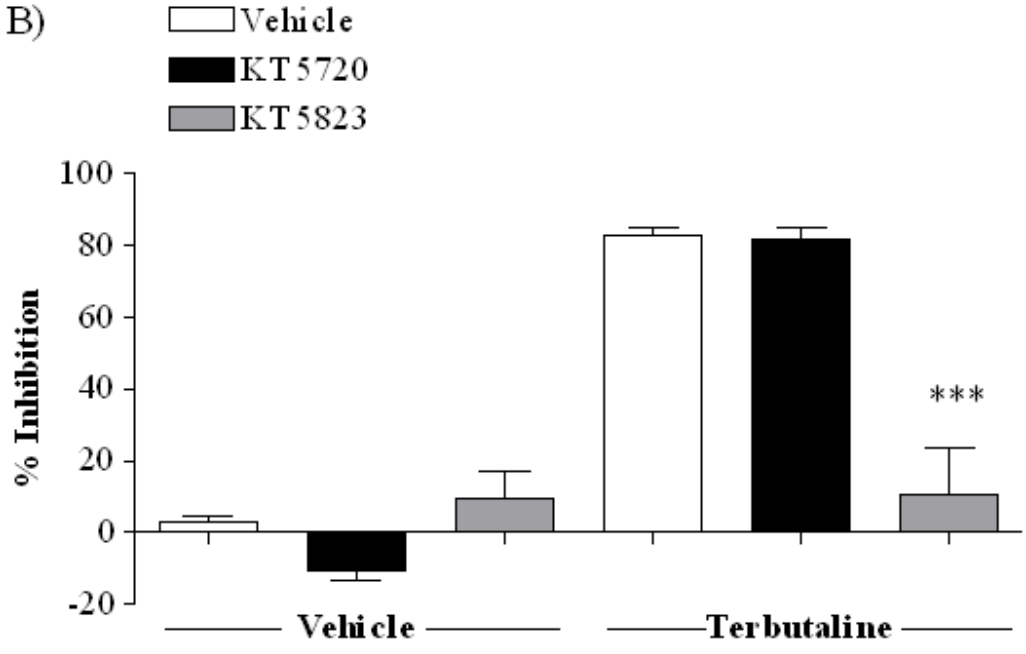


Figure 5

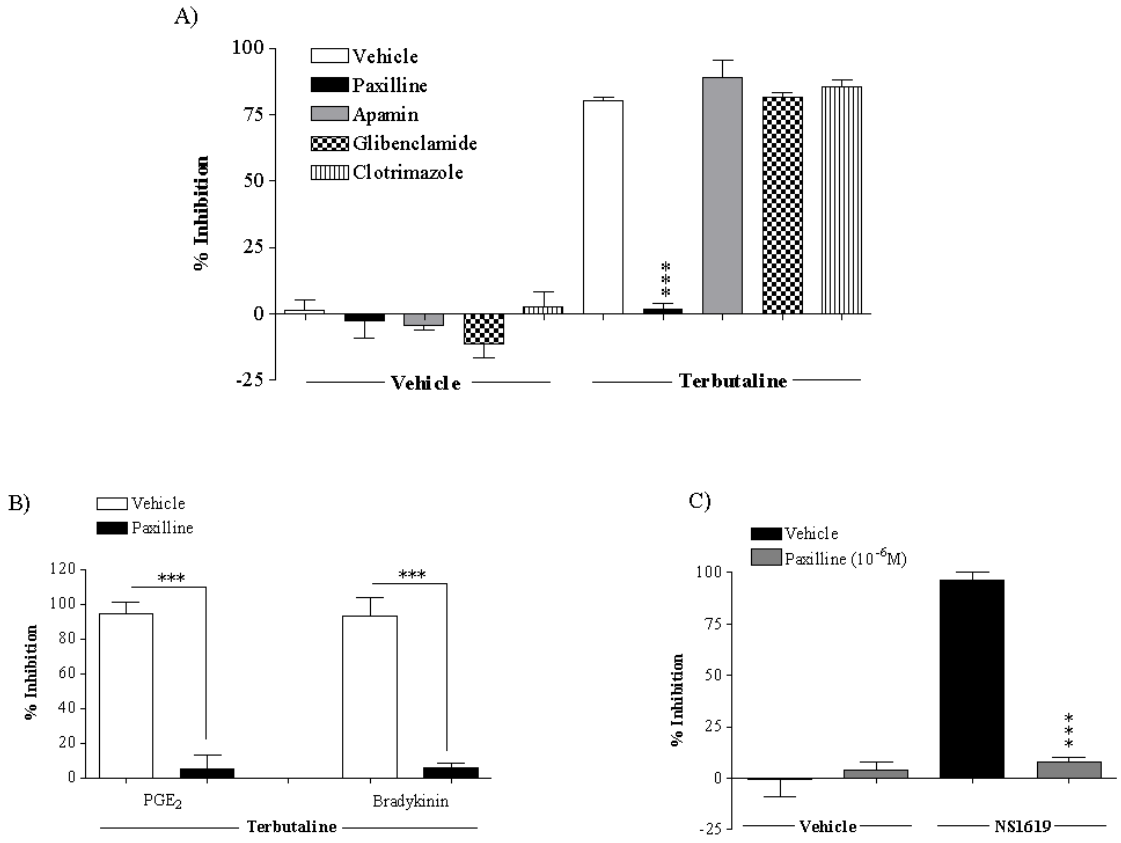
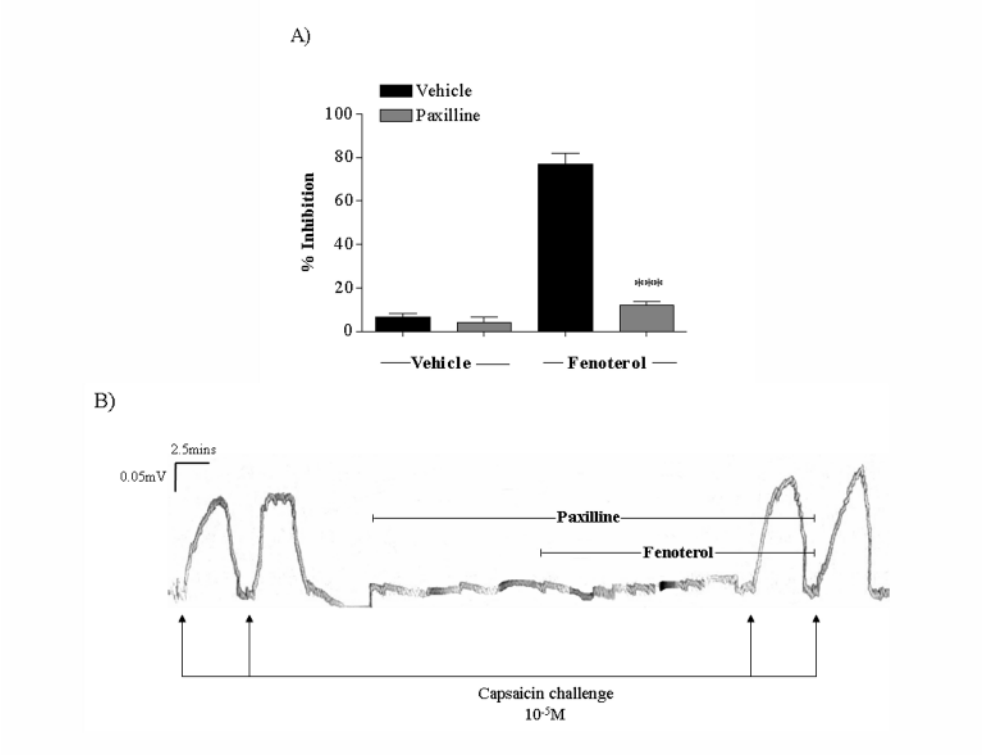

A)
Vehicle
Paxilline
Apamin
Glibenclamide
Clotrimazole
% Inhibition
100
75
50
25
0
-25
***
Vehicle
Terbutaline
B)
Vehicle
Paxilline
% Inhibition
120
100
80
60
40
20
0
***
***
PGE2
Bradykinin
Terbutaline
C)
Vehicle
Paxilline (10-6M)
% Inhibition
100
75
50
25
0
-25
***
Vehicle
NS1619

Figure 6


A)
Vehicle
Paxilline
% Inhibition
100
80
60
40
20
0
***
Vehicle
Fenoterol
B)
2.5mins
0.05mV
Paxilline
Fenoterol
Capsaicin challenge
10-5M