TUSSIVE CHALLENGE WITH ATP AND AMP: DOES IT REVEAL COUGH HYPERSENSITIVITY?

Helen Elizabeth Fowles

Hull York Medical School Centre for Cardiovascular and Metabolic Research,

Respiratory, Castle Hill Hospital, Cottingham, UK HU16 5JQ

E-mail Address: H.Fowles@hull.ac.uk

Telephone: 01482 624067

Tim Rowland

Castle Hill Hospital, Respiratory, Cottingham, HU16 5JQ, UK

Caroline Wright

Hull York Medical School Centre for Cardiovascular and Metabolic Research,

Respiratory, Castle Hill Hospital, Cottingham, UK HU16 5JQ

Alyn Morice

Hull York Medical School Centre for Cardiovascular and Metabolic Research, Respiratory,

Castle Hill Hospital, Cottingham, HU16 5JQ, UK

120 CHARACTER SUMMARY:

Chronic coughers response to ATP heightened but not sufficient to implicate as cause of cough hypersensitivity syndrome.

ABSTRACT

Recent studies have demonstrated that blockade of P2X3 adenosine triphosphate (ATP) receptors may profoundly inhibit chronic cough. We have considered whether inhaled ATP produces a tussive response and whether chronic cough patients are hypersensitive to inhaled ATP compared to healthy volunteers.

A standardised inhalational cough challenge was performed with ATP and adenosine monophosphate (AMP). 20 healthy volunteers and 20 chronic cough patients were randomised as to the order of challenges. C5 (the concentration of challenge solution causing at least 5 coughs) was compared for ATP and AMP.

The study population consisted of 6 male and 14 female volunteers in each group. 2/19 healthy volunteers coughed with AMP, none achieved C5. 8/20 chronic cough patients coughed with AMP, 2 achieved C5. 18/20 healthy volunteers coughed with ATP with 15 achieving C5. 19/19 chronic cough patients coughed with ATP, 18 achieved C5. The chronic cough patients had a greater cough response at lower concentrations of ATP.

The greater potency of ATP versus AMP in inhalational challenge suggests that tussive responses are mediated through members of the P2X purinergic receptor family. This acute effect was however not sufficient to explain cough hypersensitivity syndrome.

INTRODUCTION

Chronic cough (arbitrarily defined as a cough lasting more than 8 weeks) is a common presentation to General Practitioners and Respiratory Outpatient clinics. A recent systematic review of 90 studies found the overall global prevalence of chronic cough to be 9.6% in the general population.[1]

It is now widely accepted that, with rare exceptions, whatever the underlying aetiology of the cough, there is hypersensitivity of the vagal afferent nerves, or alteration of the central processing of their input. In hypersensitivity even trivial stimulation of these sensory nerves leads to the urge to cough. The cause of this hypersensitivity is not fully understood but the concept of Cough Hypersensitivity Syndrome (CHS) is widely agreed in the respiratory community.[2-4]

The nerves of the vagal afferent limb of the cough reflex are myelinated a-delta fibres and un-myelinated C-fibres.[5] The involvement of the terminals of these nerves in generating cough is better evidenced in animals than in humans, although recent studies suggest that similar structures may be visualised in man.[6]

The sensory receptors involved in activation of these nerve endings are of great interest as potential therapeutic targets in chronic cough. Objective demonstration of hypersensitivity may lead to the development of diagnostic tests for CHS. Chronic cough patients have been found to be hypersensitive to established cough challenges such as capsaicin[7, 8] and citric acid.[9] However the wide normal range of cough sensitivity to these agents makes them unsuitable as a diagnostic tool. Recently the demonstration that blockade of ATP preferring purinergic receptors led to a marked reduction cough frequency in chronic cough[10] suggested that ATP may be a key mediator of cough hypersensitivity and thus ATP challenge may differentiate between a normal cough reflex and cough hypersensitivity. Two classes of

purinergic receptors have been characterised: P1 and P2. The P1 receptors are activated by adenosine and AMP whereas P2 receptors respond to ATP.

To explore the hypothesis that P2 receptor activation underlies cough hypersensitivity we have compared cough challenge in two groups – healthy volunteers and patients with CHS, quantifying the cough response to AMP (P1) and ATP (P2).

METHODS

This was a randomised, controlled crossover trial of cough challenge with ATP and AMP. 20 patients with CHS were recruited from the Hull cough clinic and the Hull Respiratory Clinical Trials Unit, (CTU) database of chronic cough patients. 20 gender matched healthy volunteers were recruited from departmental staff and the CTU database of volunteers. The first participant was recruited in January 2015, the last cough challenge was completed in May 2015.

Healthy volunteers had a no evidence of cough hypersensitivity as demonstrated by a Hull Airways Reflex Questionnaire (HARQ) score of less than 13. Chronic cough patients had cough hypersensitivity as demonstrated by a HARQ score of 20 and above.

All participants were current non-smokers who had been stable on medication for at least a month. Excluded were volunteers who had a recent upper respiratory tract infection or cough/asthma exacerbation within the last 3 weeks.

Participants received two cumulative cough challenges in the CTU; one with ATP and one with AMP (Sigma Aldrich). 0.9% saline was chosen as the solvent for the ATP and AMP. AMP was used given the insolubility of adenosine in saline. ATP was readily soluble in saline at a maximum concentration of just over 0.3M. As a result, this concentration was chosen as the maximum concentration for both challenge solutions.

Stability of ATP and AMP in solution was confirmed using HPLC analysis by the University of Hull, Department of Chemistry. Both challenge solutions were found to be stable in solution for at least 72 hours. They were made up by a lab technician in single aliquots and when not used on the same day were stored in a refrigerator at $4^{o}C$.

The two challenges were administered on different days, at least 48 hours apart. The order in which the challenges were administered was randomised using a computer generated

randomisation system (Sealed Envelope). The order of administration was double-blind and the two challenge substances looked identical once made up.

The cough challenge was adapted from ERS standardised cough challenge methodology.[11] A single inhalation of each dose of challenge solution was delivered using a Ko-Ko digidoser with flow limiter, after maximal exhalation. All challenges started with a saline inhalation. ATP or AMP was then delivered in increasing concentrations on a half-log scale from 0.1-300mM. Coughs were counted by the cough challenge administrator in the first 15 seconds after each inhalation. There was at least one minute between each inhalation. Participants were asked to avoid caffeine and menthol for 1 hour prior to each cough challenge.

The challenge was terminated once the participant coughed at least 5 times following an inhalation or reached the maximal concentration available. Comments about sensation evoked during the challenge were not actively sought, but were noted if the participants volunteered them.

Participants received their second challenge at a similar time of day. If the patient experienced changes in health between the challenges these were noted, and if this was an upper respiratory tract infection, the second challenge was delayed.

Since the effects of these challenge solutions were previously unknown it was impossible to create an accurate power calculation. However, multiple previous studies done in the Hull clinical trials unit in both normal volunteers and chronic cough patients have demonstrated a significant change in C2 and C5 using 20 subjects.

The primary outcome measures were the difference in C2 and C5 (concentration of substance which elicited 2 and 5 coughs respectively) between ATP and AMP; and between healthy volunteers and chronic cough patients. Statistical analysis was performed using SPSS. Differences in number of patients reaching C2 and C5 in the ATP and AMP challenges within each group were assessed using McNemar's test for related data. For purposes of

statistical analysis during comparison between the two groups of participants, if C2 or C5 was not reached – it was set at 1000mM. Comparisons of C2 and C5 between the 2 groups (HV and CCP) were made using t-test**.**

Ethical approval was obtained to complete this study from the National Research Ethics Committee (REC reference: 14/SS/1071). The study is registered on ClinicalTrials.Gov (NCT02039999).

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RESULTS

Demographics (See Table 1)

Table 1: Demographics

	Healthy Volunteers	**Chronic Cough Patients**
Gender (Male:Female)	6:14	6:14
Age (**Median**: Range)	**43**: 23-74	**71**: 27-83
Race (Caucasian: Non-Caucasian)	18:2	19:1
FEV1 % Predicted (**Median**: Range)	**101%**: 55-121	**88%**: 57-128
Hull Cough Hypersensitivity Score (**Median**: Range)	**1.5**:0-8	**35.5**:21-52
Completed Challenges (AMP:ATP)	19:20	20:19

The Healthy Volunteer group and chronic cough patient groups were matched for gender with 14 females in each. The majority of the participants were Caucasian with 2 and 1 non-Caucasians in the healthy and patient groups respectively. The age range of the chronic cough patients was higher at 27 to 83 years versus 23 to 74 years. FEV1 as a percentage of predicted was also lower in the chronic cough group with a median of 88% versus 101%.

All patients in the Healthy Volunteer group had a HARQ score of between 0 and 8. The chronic cough patient groups HARQ scores varied between 21 and 52.

Co-morbidities

Nine of the Healthy Volunteers self-reported comorbidities. All the hypersensitivity cough patients had other co-morbidities. Gastrointestinal disturbances (both upper and lower), inflammatory disorders such as arthritis and vasculitis, hypertension and other respiratory conditions were more common in the patient group.

Medications

The patient group were taking more medications. 1 participant in each group was taking ACE-inhibitors. 1 healthy volunteer was taking anti-reflux medication compared to 11 in the cough group.

Healthy Volunteers cough challenge

2/19 healthy volunteers coughed with AMP (One healthy volunteer was not challenged with AMP due to adverse event with previous challenge). One of these achieved C2, and none achieved C5. In total throughout the AMP challenges there were only 4 coughs.
Two healthy volunteers did not cough at all in response to the ATP challenge. The remaining 18 all achieved C2 with 15 achieving C5. The difference between the ATP and AMP challenges reaching C2 and C5 was statistically significant ($p < 0.01$ for both). The results of the individual healthy volunteers cough challenges to ATP are shown in Online supplement - figure I.

Cough Hypersensitivity Patients

10 of the cough hypersensitivity patients coughed at least once in response to AMP. Of these, 8 achieved C2 and 2 achieved C5. Within individuals cough response was erratic. Having coughed at least twice patients often did not cough at higher concentrations. These findings are shown in Online supplement - figure II.

19 patients completed the ATP challenge as 1 patient withdrew prior to administration. All of these patients coughed in response to ATP and all achieved C2. One patient did not achieve C5, coughing 4 times at the 2 highest concentrations. (See Online supplement - figure III).

All patients who reached C5 did so by a concentration of 100mM. The C5 in chronic cough patients was mainly distributed between 1mM and 100mM

The two patients who achieved C5 for both AMP and ATP challenges, both achieved C5 for ATP at half a log below the C5 concentration of AMP.

The difference between C2 and C5 for ATP and AMP was statistically significant ($p < 0.01$ for both)

Whereas none of the healthy volunteers coughed in response to the initial inhalation of saline, six of the cough hypersensitivity patients did. All of these only coughed in response to saline prior to one of their challenges. This was not consistently on their first exposure to a cough challenge.

Comparing Healthy Volunteers and Hypersensitivity Cough Patients

The distribution of the C2 and C5 to ATP in healthy volunteers and patients is outlined in figure 1. Healthy volunteers and patients C2 to ATP was statistically significantly different ($p = 0.047$). This was also the case for C5 ($p < 0.01$).

The average number of coughs at each concentration of ATP for healthy volunteers and chronic cough patients is compared in Figure 2.

Adverse Events

One healthy volunteer had an episode of urticaria in the 24 hours following inhalation of the ATP challenge and was withdrawn from the study. There was one episode of wheeze following AMP in a hypersensitivity cough patient which resolved following administration of inhaled salbutamol.

One patient withdrew after their first challenge as they had felt that cough was increased in the days after the challenge.

Participants in both groups informally reported that they had throat irritation which lasted for up to several hours after the ATP challenge.

DISCUSSION

When comparing healthy volunteers with chronic cough patients, the patient group coughed significantly more, and at lower concentrations of ATP. However, the degree of hypersensitivity demonstrated by our patient group to ATP does not appear to be any more than previously seen in other cough inhalational challenges.[11-13] This suggests that chronic cough patients do not have an intrinsically heightened sensitivity to ATP and, thus it is not the acute, peripheral response to ATP that underlies the cough hypersensitivity in these patients.

The use of citric acid as a tussive challenge in humans was first described by Bickerman and Barach in 1954[14]. Since then the technique has been used in a number of different settings with a different tussive agents. The most commonly used are citric acid, capsaicin and fog challenge.

These challenges stimulate cough by acting on different peripheral nerve receptors in the airways. Capsaicin is known to stimulate TRPV1 receptors on sensory afferent C-fibres;[15] citric acid shows some cross-reactivity with a number of receptors on C-fibres and also a-delta fibres;[16] and the specific receptors stimulated by the low chlorine solution of a fog challenge are poorly defined.[17] Other receptors implicated in the cough response are more difficult to stimulate with inhalational challenge given the nature of the ligands involved, however cinnemaldehyde has been shown to cause cough in man and supports the involvement of the TRPA1 receptor in the cough reflex.[18]

One hypothesis for the cause of hypersensitivity in chronic cough patients is that there is an up-regulation in one or more of the peripheral receptors of the afferent limb of the cough reflex.[19] The blockade of receptors stimulated by cough challenges are therefore of interest as therapeutic targets. In animal studies, there has been some success in reducing cough by

antagonising these receptors.[20, 21] However, this success has not been replicated in human trials of TRPA1 (personal communication) and TRPV1 receptors,[22] suggesting that these afferent sensory receptors which are undoubtedly important in the sensation of irritation stimulating cough are however not the root cause of the cough hypersensitivity.

A number of different lines of evidence now point to the purinergic system as being the most likely mechanism for inducing afferent hypersensitivity. ATP as an extracellular signaling molecule, acting through a class of purinergic receptors, was first postulated by Burnstock in 1972.[23] It took a number of years for this mechanism to become established and the role of ATP as an extracellular signal was not widely accepted until the 1990's when the first purinergic receptors were cloned. [24, 25]

A number of findings support the presence of ATP responsive P2 receptors within mammalian lungs.. P2X3 receptors are thought to be mainly responsible for the effects of ATP in the lung having been demonstrated by immunohistochemistry on sensory nerve endings.[26] It is found as either a homotrimeric P2X3 receptor or the heterotrimeric P2X2/3 [27] Vagal C-fibres may be stimulated by ATP via heteromeric P2X2/3 receptors.[28] Responses of peripheral neurons to ATP vary within the same ganglia, between different types of ganglia and within species. However the response appears to be consistently due to its effect on P2X2 and P2X3 but there is probably a difference in the proportion of homo/hetero types of receptor expressed.[27] Of relevance to the cough hypersensitivity is the observation that activation of P2X2/3 heterodimers produces a prolonged current, where stimulation of P2X3 receptors produces a rapidly inactivating current.[29] A recent study has however suggested that prolonged activation of the P2X3 receptor may be achieved by TRPV4 activation of pannexin causing the continuous stimulation of P2X3, thus leading to prolonged hypersensitivity.[30]

Animal studies considering the role of ATP and P2X receptors in cough have been limited to guinea pigs where inhaled ATP accentuated subsequent citric acid challenge. ATP alone failed to stimulate cough and antagonist studies implicated the P2X4 receptor in this species.[31]

In man intravenous ATP administered to palliative care patients caused breathlessness as its most common side effect.[32] By inhalation ATP has previously been noted to cause cough although this was not systematically characterised. Prolonged inhalation of ATP caused bronchoconstriction in both healthy and asthmatic volunteers, with a greater response in the asthmatics.[33, 34] Inhalation challenges using ATP and AMP in COPD patients, smokers and healthy volunteers found that ATP appeared to cause increased breathlessness and cough compared to AMP.[35]

The lack of a significant cough response to inhaled AMP in our healthy participants seems to be in contrast with previous studies. This may be due to methodological differences given the brief exposure of our participants consequent on the use of the single breath inhalation method. Whilst the majority of our healthy volunteers coughed with ATP two did not. This is in keeping with the experience of other cough challenges such as citric acid and capsaicin, where a proportion of healthy volunteers do not cough within the range of the challenge. ATP challenge does not appear therefore to be exceptional in its sensitivity or persistence. Thus while our findings support the importance of purinergic receptors in the normal cough reflex pathway, it is not possible to differentiate between the P2X3 and P2X2/3 as the main modulator of this tussive response to ATP.

AF-219 (a P2X3 receptor antagonist) *(Afferent pharmaceuticals, 2755 Campus Dr, San Mateo, CA 94403, United States)* has been trialled in a phase 2 study and has been found to dramatically reduce 24 hour cough counts after 2 weeks administration in patients with chronic cough.[10] Our data would tend to support the hypothesis that while P2X3 receptor

activation may be the final common pathway producing hypersensitivity, acute activation of the receptor does not infer this state on afferent nerves and that other mechanisms such as activation of TRPV4 / pannexin are required.

Our study has several limitations. Our two study populations were gender matched because of the known influence of gender on cough reflex sensitivity. They were however not age matched and our patient group was older than our healthy volunteers. Although chronic cough is more prevalent in older patients,[36] this does not seem to be an influence of the cough reflex rather than an increased prevalence of the underlying cough provoking conditions in the elderly. Previous studies have shown no influence of age as opposed to morbidity such as dementia on the cough reflex.[37]

Co-morbidities were also more prevalent in the patient group. The majority of these are conditions which are often associated with cough, such as GORD, asthma and post nasal drip as well as irritable bowel syndrome and lymphoedema. These co-morbidities were self-reported by patients. One participant in each group were taking ACE-inhibitors, and whilst this may have influenced cough threshold these individuals did not appear as outliers.

Within the population of 40 participants, we only had one adverse effect to ATP. This urticarial rash appears to have been a hypersensitivity reaction.

Given that some of our participants commented on ongoing throat irritation after the challenge was completed, ATP may potentially be involved in cough hypersensitivity via other mechanisms than a direct pro-tussive effect.

CONCLUSION

We believe that this is the first study to compare objective cough response to inhaled ATP and AMP in healthy volunteers and chronic cough patients. The response to ATP in chronic

cough appears to be heightened, but not to such a degree to implicate the acute response to inhalation of ATP in the pathophysiology of cough hypersensitivity syndrome.

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Captions for Figures:

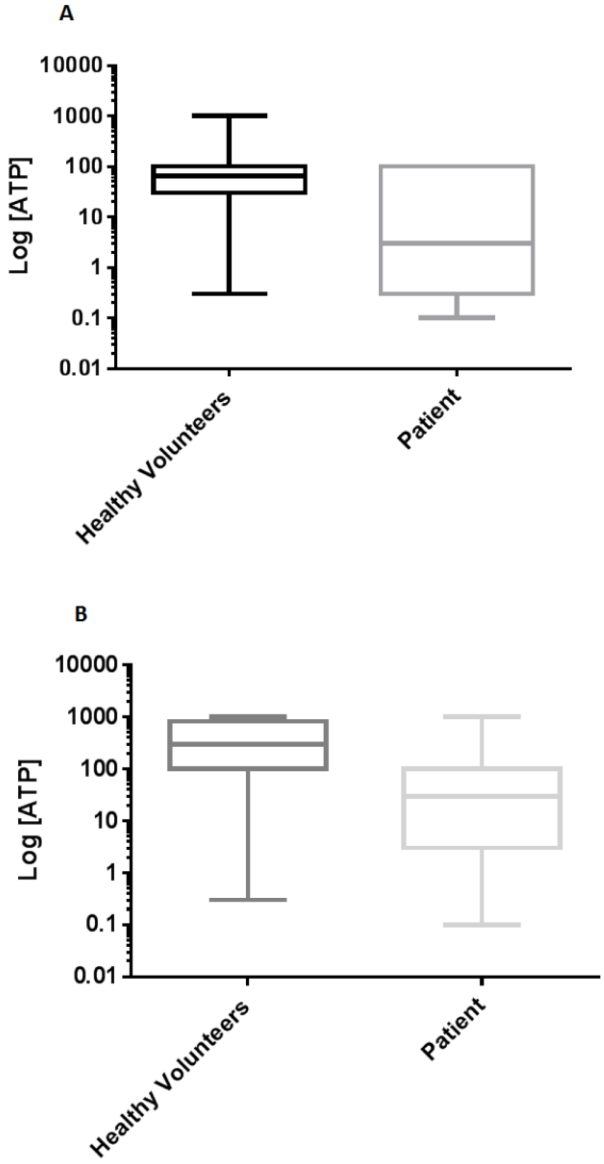
Figure 1: Box and whisker plots showing distribution of C2 (A) and C5 (B) during adenosine triphosphate challenge in healthy volunteers compared to chronic cough patients. C2, the concentration of ATP causing at least 2 coughs; C5, The concentration of ATP causing at least 5 coughs

Figure 2: Comparison of mean coughs to each concentration of adenosine triphosphate in healthy volunteers and chronic cough patients.

Online supplement - figure I: Number of coughs at each concentration of adenosine triphosphate in 20 individual healthy volunteers. N/S: Normal Saline, ATP: adenosine triphosphate

Online supplement - figure II: Number of coughs at each concentration of adenosine monophosphate in 20 chronic cough patients. N/S: Normal Saline, AMP: adenosine monophosphate

Online supplement - figure III: Number of coughs at each concentration of adenosine triphosphate in 19 chronic cough patients. N/S: Normal Saline, ATP: adenosine triphosphate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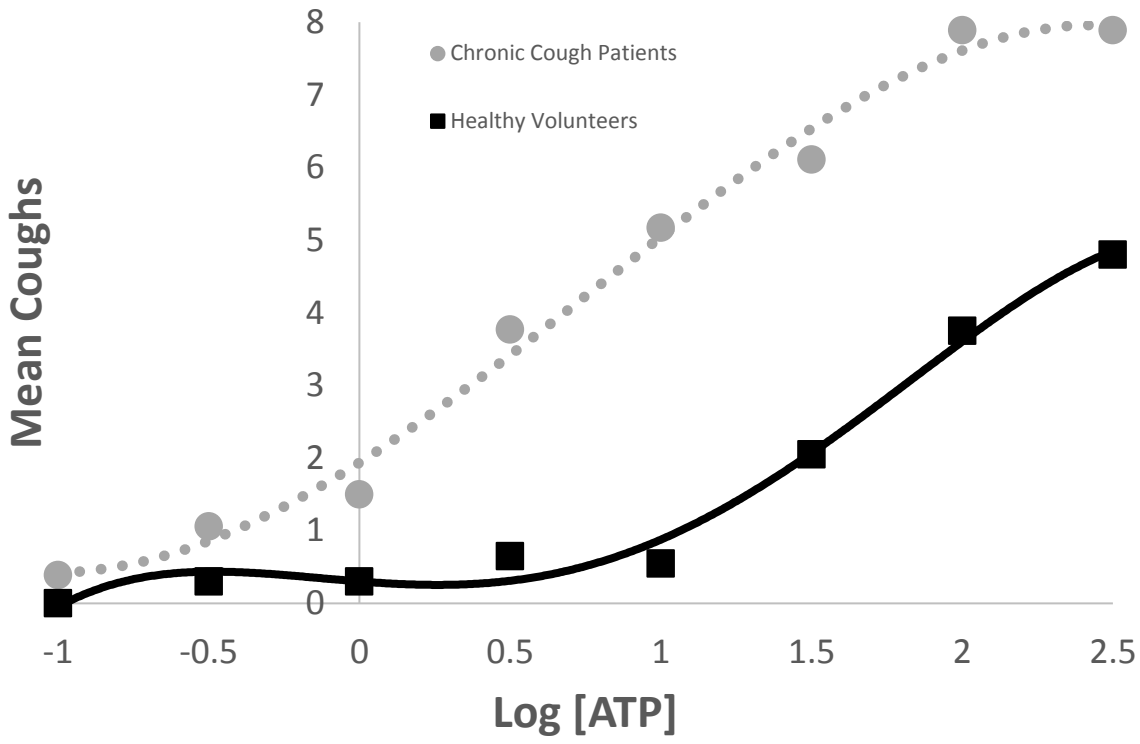
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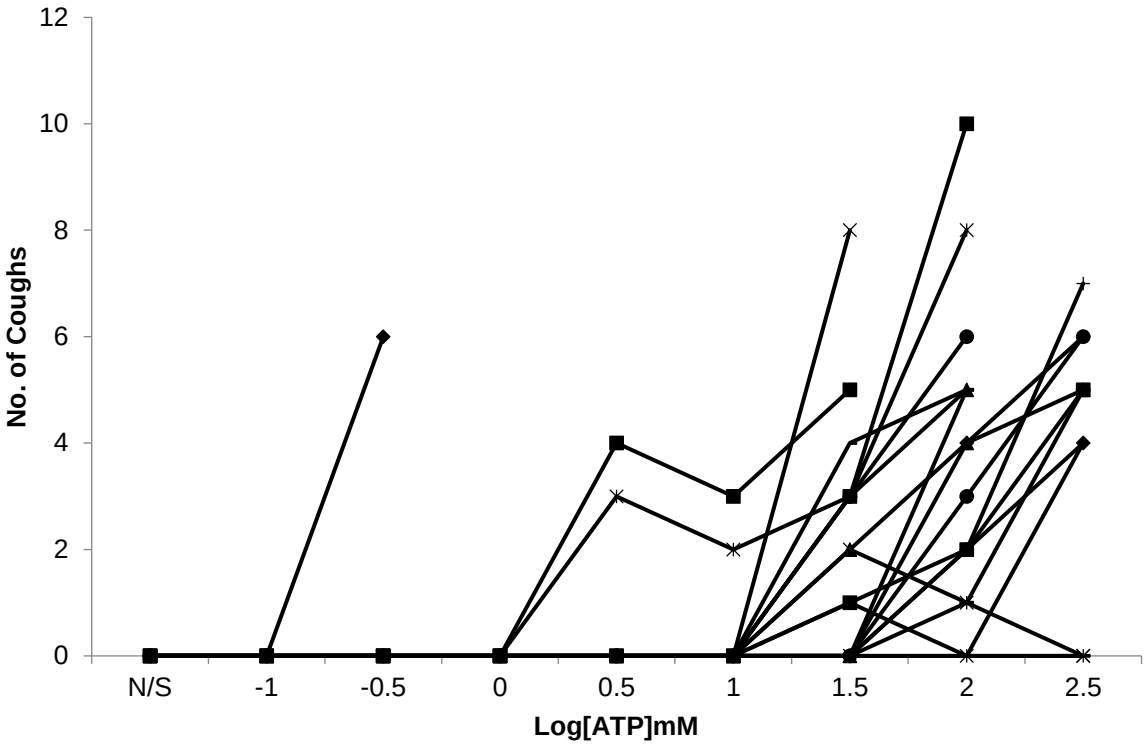
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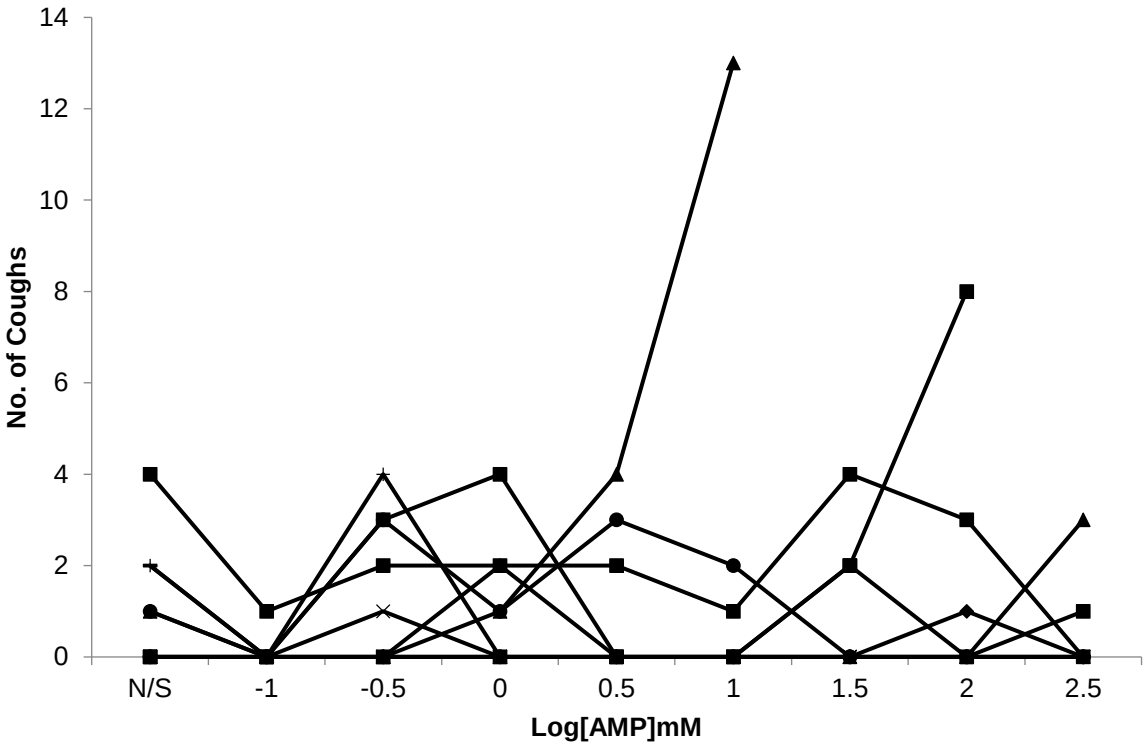
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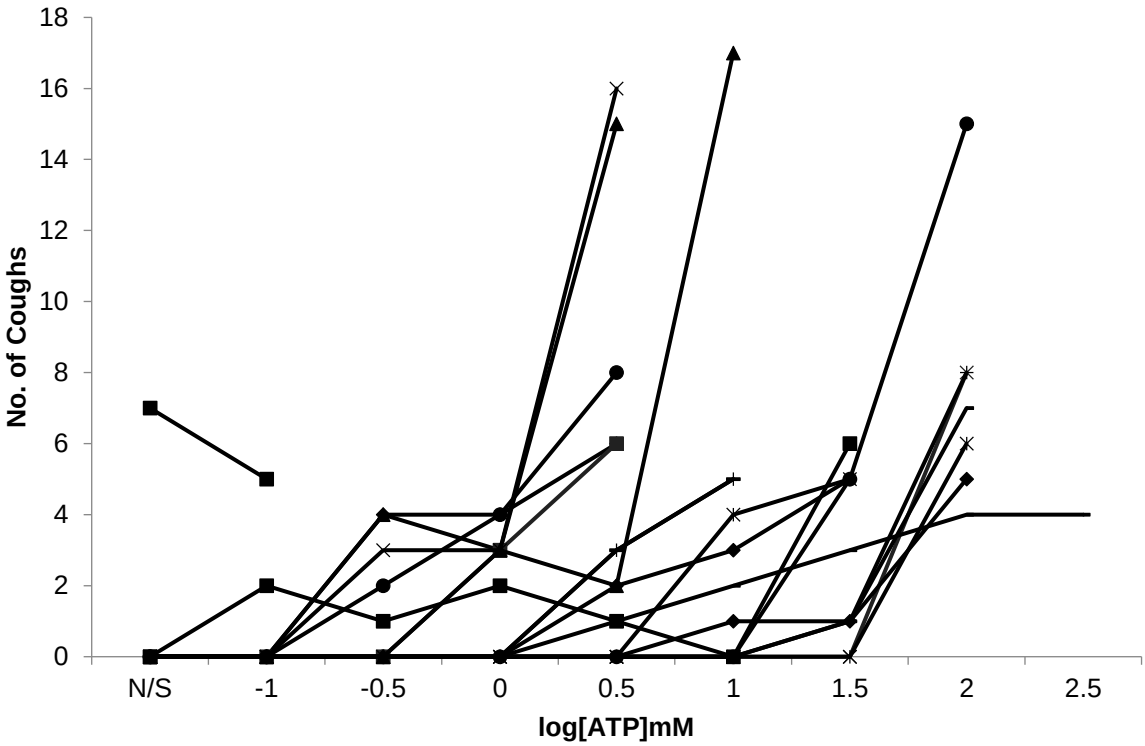
No. of Coughs
N/S
-1
-0.5
0
0.5
1
1.5
2
2.5
Log[AMP]mM


18
16
14
12
10
8
6
4
2
0
No. of Coughs
N/S
-1
-0.5
0
0.5
1
1.5
2
2.5
log[ATP]mM