

The FASEB Journal express article 10.1096/fj.04-1990fje. Published online November 17, 2004.

Theobromine inhibits sensory nerve activation and cough

Omar S. Usmani,^{*} Maria G. Belvisi,[†] Hema J. Patel,[†] Natascia Crispino,[†] Mark A. Birrell,[†] Márta Korbonits,[‡] Dezső Korbonits,[§] and Peter J. Barnes^{*}

^{*}Department of Thoracic Medicine, National Heart and Lung Institute, Imperial College London, London, United Kingdom; [†]Respiratory Pharmacology Group, National Heart and Lung Institute, Imperial College London, London, United Kingdom; [‡]Department of Endocrinology, St. Bartholomew's Hospital, London, United Kingdom; [§]Chinoin Co. Ltd., Budapest, Hungary

Corresponding author: Professor Maria G. Belvisi, Respiratory Pharmacology Group, Department of Airway Diseases, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY. E-mail: m.belvisi@imperial.ac.uk

ABSTRACT

Cough is a common and protective reflex, but persistent coughing is debilitating and impairs quality of life. Antitussive treatment using opioids is limited by unacceptable side effects, and there is a great need for more effective remedies. The present study demonstrates that theobromine, a methylxanthine derivative present in cocoa, effectively inhibits citric acid-induced cough in guinea-pigs *in vivo*. Furthermore, in a randomized, double-blind, placebo-controlled study in man, theobromine suppresses capsaicin-induced cough with no adverse effects. We also demonstrate that theobromine directly inhibits capsaicin-induced sensory nerve depolarization of guinea-pig and human vagus nerve suggestive of an inhibitory effect on afferent nerve activation. These data indicate the actions of theobromine appear to be peripherally mediated. We conclude theobromine is a novel and promising treatment, which may form the basis for a new class of antitussive drugs.

Key words: vagus • methylxanthines

Cough is a protective, primitive reflex, in healthy individuals (1). However, when cough serves no useful role, it is the most common respiratory complaint for which medical attention is sought (2). Persistent cough can be debilitating, socially distressing, and adversely impairs quality of life (3). Cough leads patients to use over-the-counter remedies as first-line treatments; in the United States alone, sales for these over-the-counter remedies exceed \$2 billion dollars (4). A recent meta-analysis, however, established that evidence regarding the effectiveness of such remedies was inconclusive (5).

Narcotic agents with a morphine skeleton, such as the opioids codeine and dextromethorphan, are the most widely used antitussives in cough remedies, but they have unpredictable efficacy and undesirable central nervous and peripheral side effects that often lead to their discontinuation (6, 7). The 2nd International Cough Symposium concluded that there is a great need for effective

new cough treatments, as well as a better understanding of the complex genesis and pathophysiology of cough to guide the development of pharmacological approaches (8).

The cough reflex is initiated by stimulation of two different classes of sensory afferent fiber, namely the myelinated rapidly adapting receptors (RAR) and nonmyelinated C-fibers with bronchial or pulmonary endings (9, 10). Inappropriate activation of these nerves can occur in allergic diseases (e.g., asthma) and chronic obstructive pulmonary disease (COPD) and lead to many of the symptoms such as coughing. However, the mechanisms involved in the abnormal functioning of airway nerves have not yet been described. They are thought to involve the release of inflammatory mediators which sensitize the nerve fibers leading to increased electrical activity of these fibers and an increase in the release of various neurotransmitters from the nerve endings (6).

We hypothesize that agents that inhibit sensory nerve activity, that is nerve depolarization, will also inhibit the cough reflex. Although many compounds demonstrate promising characteristic antitussive effects in animal models, few have shown any clinical benefit (11). In this paper, we describe theobromine, a methylxanthine alkaloid derivative predominant in cocoa, as a novel and promising therapy for the treatment of cough. Theobromine was developed alongside other methylxanthines for respiratory disorders, but disappeared from clinical use because of its low potency as a bronchodilator (12). Recent studies, however, demonstrate a unique antitussive effect, unlike the other methylxanthines, in a series of pharmacological studies in the guinea pig cough model using a synthetic analog (13, 14).

We, therefore, sought to investigate the action of theobromine in the guinea pig cough model in vivo, and in isolated human and guinea-pig vagus nerve preparations in vitro. We observed theobromine inhibited cough in our human trial at concentrations that do not have central side effects in man and thus is unique in the field of cough therapy. Furthermore, our data show the antitussive mechanism of action is probably due to direct inhibition of sensory nerve activation. Our study reveals a promising prospect for a potentially new antitussive that is acutely needed in the clinical arena for treatment of patients with both acute and chronic cough.

METHODS

In vivo animal experiments were carried out according to the Institutional Guidelines for Care and Use of Experimental Animals and approved by the animal Ethics Committee of Chinoïn (Chinoïn Co. Ltd., Budapest, Hungary). For the human studies, written informed consent was obtained from all patients, and approved by the Ethics Committee of the Royal Brompton and Harefield Hospital National Health Service Trust (London, United Kingdom).

Citric acid-induced cough in the guinea pig

Cough was induced in conscious guinea pigs by a previously described method (14). Briefly, female Dunkin Hartley guinea pigs (250–300 g) were individually placed in transparent chambers and exposed to 0.78 M citric acid aerosol solution for 3 min. Coughs were counted by a trained observer and recognized from the characteristic opening of the mouth and posture of the animal. Animals with six or more coughs were selected and orally dosed with theobromine or codeine in a suspension of 0.1% methylcellulose vehicle (treatment groups) or with 0.1%

methylcellulose alone (control group). A second citric acid exposure was evoked one hour after dosing in dose–response experiments, and at 0.5, 1, 2, 3, 4, and 6 h in time-duration experiments. The antitussive activity was calculated as the percentage decrease of the number of coughs between the second and the first challenge. The drug-treated groups were compared with the control group using the Kruskal-Wallis test followed by the Conover Inman test for pair-wise comparisons in the dose–response experiments, or by using the Student's *t* test in the time-duration experiments (Graph Pad Prism Software, San Diego, CA). Statistical significance was taken at $P < 0.05$.

Capsaicin cough challenge in human subjects

Ten healthy nonsmoking subjects (mean age (\pm SD) 38 ± 8.1 year, six females) participated in a randomized double-blind crossover study. The antitussive effect of a single-dose of theobromine (1000 mg) was compared with codeine phosphate (60 mg) and placebo during three study visits, each separated by a washout period of a week. The dose of theobromine was determined in a pilot study. Interestingly, a previous study documented a bronchodilator effect of theobromine within a similar dose range (at 500–1000 mg) in patients with asthma (12). The main outcome measure was the capsaicin concentration required to induce five coughs (C5). The capsaicin inhalation challenge was performed according to a modified version of our previously published protocol (15). Briefly, subjects inhaled nebulized single-dose doubling concentrations (0.5–500 μ M) of capsaicin delivered via a breath-activated dosimeter (P. K. Morgan Ltd., Gillingham, UK), at one-minute intervals. Capsaicin doses were alternated at random with 0.9% saline doses to minimize conditioned responses during the study. Coughs were counted for 1 min after the inhalation by direct observation. The C5 was determined, and the capsaicin dose was repeated for confirmation. The C5 was obtained on two screening occasions, and subjects were eligible to enter the study if their C5 was in the middle of the dose response (up to ≤ 31 μ M) and reproducible to ≤ 1 doubling difference between both screening visits. Subjects were asked to refrain from tea, coffee, chocolate, and caffeine-like substances 12 h before each visit. At each treatment visit, the C5 threshold was determined two hours postdosing, which coincides with the plasma peak of theobromine (16). Subjects were questioned about adverse effects, including cardiovascular, central nervous, and gastrointestinal symptoms. Individual capsaicin C5 data was logarithmically transformed to normalize the data, and the means were used to test the null hypothesis using two-way ANOVA, with factors for subject and treatment (Graph Pad Prism Software). Modified *t* tests to compare subsequent groups of interest were undertaken using the Bonferroni method to adjust for the *P* values and multiple comparisons. Statistical significance was taken at $P < 0.05$.

Measurement of sensory nerve depolarization in isolated vagus nerve preparations

Male Dunkin-Hartley guinea pigs (300–350 g) housed in a temperature-controlled (21°C) room with food and water freely available, were killed by cervical dislocation, and the vagus nerves caudal to the nodose ganglion were carefully dissected and placed in oxygenated Krebs–Henseleit solution (KHS) of the following composition (mM): NaCl - 118; KCl - 5.9; MgSO₄ - 1.2; NaH₂PO₄ - 1.2; CaCl₂ - 2.5; glucose - 6.6; NaHCO₃ - 25.5; and bubbled with 95% O₂ and 5% CO₂ (BDH, Poole, UK). Human trachea, with branches of the cervical vagus still attached, was obtained from a patient (male 44 year with emphysema) undergoing heart-lung transplantation. Both human and guinea pig vagus nerve trunk segments (40–50 mm) were

placed in oxygenated KHS, carefully desheathed, and mounted in a 'grease-gap' recording chamber as described previously (17, 18). Briefly, each nerve was drawn longitudinally through a narrow Perspex channel and petroleum jelly applied at the center of the channel creating an area of high resistance, and electrically isolating the extracellular space between the two ends of the nerve. Nerves were constantly superfused with KHS at 37°C. Silver (Ag/AgCl) electrodes (Mere 2 Flexible reference electrodes, World Precision Instruments, Stevenage, UK), made contact at each nerve trunk end, and recorded DC potential and nerve depolarization via a DAM 50 differential amplifier (WPI) onto a calibrated pen-chart recorder (Lectromed Multi-Trace 2, Letchworth, UK). Control sensory nerve depolarizations were induced by perfusion of the vagus nerve with a pre-established submaximal concentration of capsaicin (1 μ M in guinea pig) (18) and (10 μ M in human) (18) applied for 4 min, after which the tissue was washed until the baseline response of the nerve was regained. After two reproducible control responses were obtained to capsaicin, preparations were then perfused for 20 min with theobromine (0.01–100 μ M), codeine (0.01–100 μ M), or the relevant vehicle control (0.1% DMSO), following which capsaicin (1 μ M) was reapplied in the continued presence of the test agents. Experiments were randomized so that different concentrations of different drugs were tested on vagus nerves from the same animal on the same day. The data were subjected to a paired two-tailed *t* test since the response to a stimulant was measured before and after drug intervention within the same nerve. pD_2 values ($-\log$ of the EC_{50} defined as the concentration of drug required to elicit 50% of the maximum inhibition) and statistical significance, taken at $P < 0.05$, were calculated using 'GraphPad InStatTM' (Graph Pad Prism Software).

RESULTS

Theobromine as a potential antitussive

Several synthetic antitussives are characterized by the presence of a 1,2,4-oxadiazole ring in their chemical structure (Fig. 1A). With the 'renaissance' of the methylxanthine, theophylline (19), for the treatment of asthma in the seventies, a series of novel compounds with an oxadiazolylalkyl substituent at the N7 atom on the basic xanthine skeleton (Fig. 1B), were synthesized (Fig. 1C), and investigated as potential antiasthmatic and antitussive agents.

With two of these compounds selected for preclinical testing, 3,7-dihydro-3-methyl-7-/(5-methyl-1,2,4-oxadiazol-3-yl)methyl/-1*H*-purine-2,6-dione (Fig. 1D) (14, 20), and 3,7-dihydro-1,3-dimethyl-7-/(5-methyl-1,2,4-oxadiazol-3-yl)methyl/-1*H*-purine-2,6-dione (Fig. 1E) (13), there was an unexpected correlation between chemical structure and antitussive potency. When the N1 atom of the xanthine skeleton remained unsubstituted, the antitussive effect of these 1*H*-xanthine derivatives increased considerably compared with the corresponding N1-methyl analogs. The antitussive effect of 3,7-dihydro-3-methyl-7-/(5-methyl-1,2,4-oxadiazol-3-yl)methyl/-1*H*-purine-2,6-dione was about eightfold stronger than that of 3,7-dihydro-1,3-dimethyl-7-/(5-methyl-1,2,4-oxadiazol-3-yl)methyl/-1*H*-purine-2,6-dione (14). This correlation among the synthetic xanthine derivatives suggests that if the association also applies to the three natural methylxanthine alkaloids, theobromine (Fig. 1F), theophylline (Fig. 1G) and caffeine (Fig. 1H), then theobromine, which in contrast to theophylline and caffeine is unsubstituted at N1, should exhibit a therapeutically significant antitussive effect.

Theobromine inhibits citric acid-induced cough in the guinea pig model

We investigated the antitussive effect of theobromine on citric acid-induced cough in guinea pigs, using codeine hydrochloride as a positive control. Cough provocation in conscious guinea pigs is widely used to test the efficacy of new cough treatments (21, 22). Theobromine and codeine showed a dose-dependent antitussive effect on citric acid-induced cough in guinea pigs compared with the vehicle-treated control groups ([Fig. 2](#)). The antitussive effect of theobromine and codeine was similar to each other, but significantly better than vehicle treatment using doses between 4–64 mg/kg and 8–64 mg/kg, respectively. Vehicle treatment itself caused some decrease in the numbers of citric acid-induced cough, which were similar within the two treatment groups ($P=0.92$) ([Fig. 2](#)). Theobromine at a dose of 32 mg/kg produced a long-lasting antitussive effect that was statistically significant for up to 4 h after treatment ([Fig. 3](#)). There was no tachyphylaxis evident in the tussive response elicited by citric acid as can be seen following repeat exposure to the tussive agent, following vehicle administration, in the time-course experiment ([Fig. 3](#)).

Theobromine inhibits capsaicin-induced cough in man

We next examined whether theobromine could inhibit induced cough in human subjects. Capsaicin is a well-established, reproducible, sensory C-fiber stimulant in vitro, widely used as a tussive stimulant in clinical research to determine the cough threshold (23). In 10 healthy volunteers, theobromine significantly increased the capsaicin concentration required to induce five coughs (C5, cough threshold) when compared with placebo ($P<0.01$) ([Fig. 4](#)). The log C5 (\pm SD) was 1.43 ± 0.65 , 1.59 ± 0.74 , and 1.86 ± 0.58 for placebo, codeine, and theobromine, respectively. There was no significant difference between codeine and placebo ($P=0.25$). No adverse effects, particularly cardiovascular or central nervous, were observed.

Theobromine inhibits capsaicin-induced sensory nerve depolarization of guinea pig vagus and human vagus nerves in vitro

To ascertain whether the mechanism of action was peripheral or central, the effect of theobromine ([Fig. 5A](#)) and codeine ([Fig. 5B](#)) on capsaicin-induced guinea pig vagus nerve depolarization was investigated. Perfusion of guinea pig vagus nerve preparations with theobromine (0.01–100 μ M) inhibited capsaicin-induced nerve depolarization in a concentration-dependent manner ($pD_2=5.2$). Maximum inhibition of $94.9 \pm 3.8\%$ was observed at the highest concentration used ([Fig. 5A](#)). Similarly, nerve preparations treated with codeine (0.01–100 μ M) showed markedly reduced sensory nerve depolarization induced by capsaicin in a concentration-dependent manner ($pD_2=5.6$), and complete inhibition of induced nerve depolarization was achieved at 100 μ M ([Fig. 5B](#)).

We also studied the effects of theobromine on depolarization of a human vagus nerve preparation by capsaicin. Human vagus preparations were used to confirm the observations seen with guinea pig vagus nerves to provide the appropriate validation of the target in man and confirm clinical relevance. Theobromine (100 μ M) inhibited capsaicin (10 μ M)-induced nerve depolarization of the human vagus by 66.7% ([Fig. 6](#)).

DISCUSSION

We have demonstrated for the first time that theobromine, a methylxanthine derivative present in cocoa, effectively inhibited citric acid-induced cough in conscious guinea pigs *in vivo*, and using a randomized double-blind study in healthy human volunteers, we showed that theobromine inhibited capsaicin-induced cough. We also established by using isolated guinea pig vagus *in vitro* that the antitussive mechanism of action probably involves direct inhibition by theobromine of capsaicin-induced sensory nerve activation. Furthermore, we demonstrated the inhibitory action of theobromine on capsaicin-induced nerve depolarization using an isolated human vagus nerve preparation, providing *in vitro* proof of concept for this action in man. However, further confirmation of this observation is needed as it was only possible to study one human vagus sample in the course of these studies.

Even in their recognized clinical role as bronchodilators, the underlying mechanism of action by methylxanthines is unclear, but is most likely related to their phosphodiesterase inhibitory activity, and also antagonism of bronchoconstrictor adenosine A₁ receptors (20, 24). Prostaglandins PGI₂ and PGE₂ cause cyclic AMP accumulation in peripheral nerves (25) and although cAMP-elevating drugs have recently been shown to activate sensory C-fibers in the isolated rat vagus *in vitro* (26) little is known regarding the neuromodulatory role of cyclic nucleotides and adenosine on human sensory nerve function. Interestingly, methylxanthines have recently been shown to activate human intermediate-conductance, Ca²⁺-activated K⁺ channels (hIK) (27). We have previously proposed that activation of large conductance Ca²⁺-activated potassium channels might be a common mechanism of G-protein coupled receptor activation leading to inhibition of afferent and efferent vagal activity (28), but a role for the hIK channel in the modulation of sensory nerve activity has not yet been demonstrated.

To determine the mechanism of action of theobromine, we determined the effect of theobromine on the isolated guinea pig vagus nerve preparation, which is a validated *in vitro* model that allows evaluation of compounds on sensory nerve depolarization and appears predictive of data obtained in single sensory fiber recording experiments (18, 28). We previously demonstrated that both guinea pig and human vagus isolated nerve preparations respond similarly to a variety of tussive sensory nerve stimulants, including capsaicin, suggesting that human vagal afferent fibers react to and show similar responses to afferent stimulants acting on guinea pig nerves (M. Belvisi, unpublished data). Our data demonstrate that guinea pig and human vagus nerves respond similarly to theobromine inhibition of capsaicin-induced depolarization. We have previously demonstrated the utility of the isolated vagus preparation as a preclinical *in vitro* screen, which can be used to predict the efficacy of test compounds before evaluation in the *in vivo* guinea pig cough model. Furthermore, the data obtained in the guinea pig cough model was similar to that obtained in the clinical studies in agreement with published studies describing similarities in the responsiveness of the cough reflex between the two species (22). Taken together, the findings with theobromine in the guinea pig and human cough models *in vivo*, as well as in the guinea pig and human nerve preparations *in vitro*, are consistent with the hypothesis that the antitussive action was suggestive of a direct inhibition of sensory nerve activation rather than by a centrally mediated mechanism. Although the evidence presented suggests that theobromine may inhibit sensory nerve function via a peripheral mechanism, no evidence has been presented suggesting the absence of a centrally mediated effect and as such, this remains to be confirmed. However, although the isolated vagus preparation presents us with

the ideal opportunity to conduct a comprehensive pharmacological assessment, data using this preparation should be interpreted with some caution as the pharmacological agents are applied to the axon of the isolated vagus nerve in vitro. Thus, the depolarization signal recorded extracellularly represents a summation of the changes in membrane potential of all the axons via activation of receptors expressed in the neuronal membrane of the axon. Conduction velocities of the fibers are not recorded and action potentials per se are not measured, so identification of the activation/inhibition of specific populations of sensory nerve fiber is not possible. Furthermore, the receptor expression and signal transduction mechanisms in the axon may not necessarily represent the behavior of those elements in the peripheral endings.

In our human study, no adverse effects were observed of the cardiovascular or central nervous system, and these data support the use of theobromine as an effective antitussive agent in man, with a safe therapeutic index. We compared theobromine to codeine phosphate, often used as a benchmark against which new cough treatments are compared (29), and although systemic opiates suppress capsaicin-induced cough (30), they are associated with many unacceptable adverse effects when used in effective antitussive doses (7). To achieve maximum sensitivity in the capsaicin cough challenge methodology, we controlled the capsaicin variability in our study population by selecting individuals with no more than one difference in the doubling concentration of capsaicin (31). Subjects in the middle of the cough dose–response were selected, and those with attenuated or hyperresponsive effects were excluded to obtain reproducible results. Our population was biased toward women, supported by previous studies observing greater capsaicin sensitivity in healthy women (32).

In conclusion, the data described demonstrate a significant antitussive effect of theobromine in healthy subjects when compared with placebo. Further studies are needed to see whether these effects can be extrapolated to patients with chronic persistent cough, who, interestingly, have previously been demonstrated to exhibit increased sensitivity to the tussive effect of the inhaled irritant, capsaicin (33, 34). These data suggest that theobromine could form the basis for development of novel and safe antitussive agents for the treatment of a very common and troublesome symptom.

ACKNOWLEDGEMENTS

We are grateful for the help of Endre G. Mikus, Péter Arányi, and István Jelinek (Chinoin Co. Ltd., Budapest, Hungary).

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Received June 25, 2004; accepted October 12, 2004.

Fig. 1

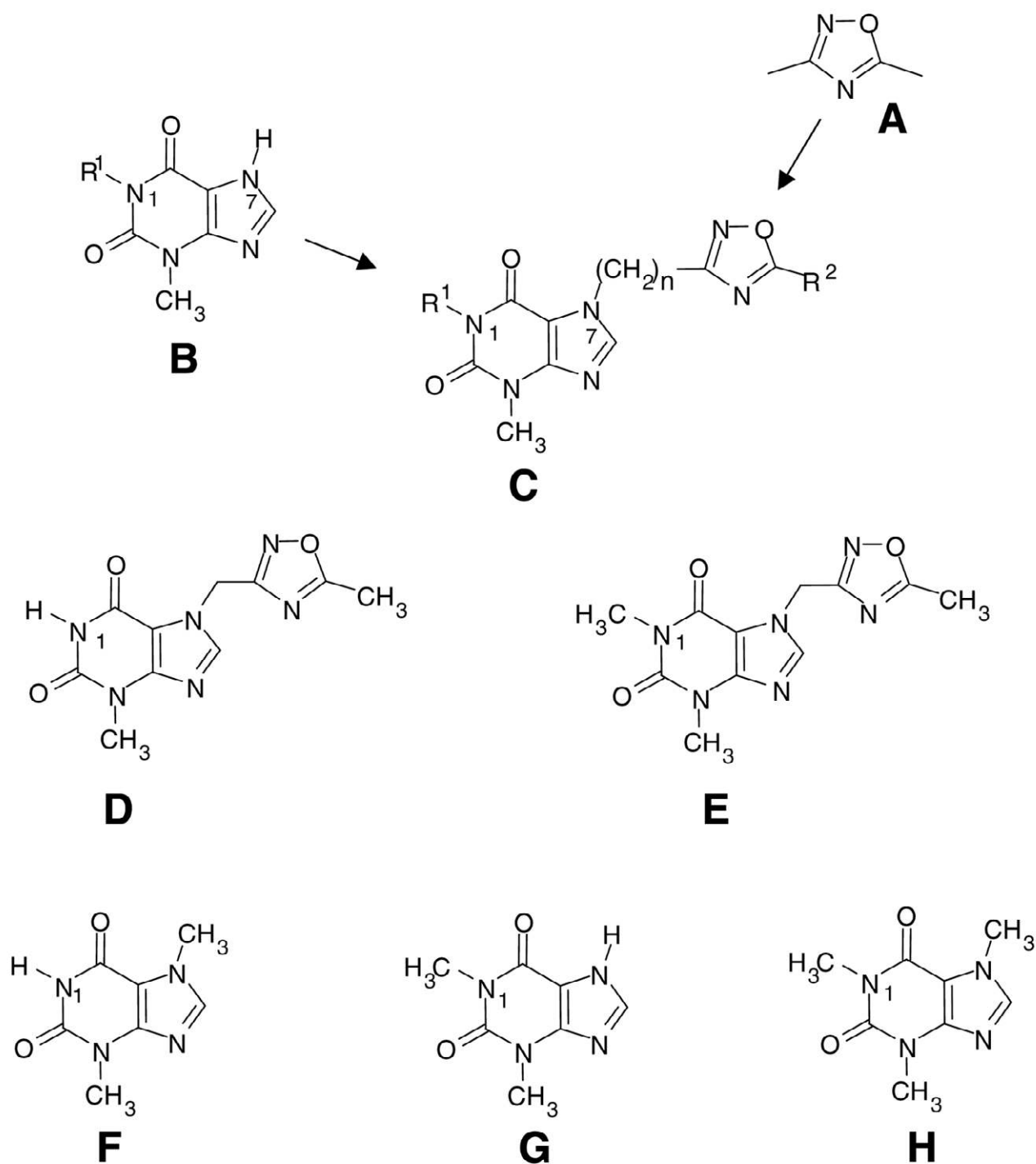


Figure 1. Chemical structures of the xanthine derivatives. Synthetic antitussives have a characteristic 1,2,4-oxadiazole ring (A), and incorporating this into the N7 atom of the basic xanthine skeleton (B), a series of novel compounds were synthesized (C–E). The antitussive activity of the N1 unsubstituted compound, 3,7-dihydro-3-methyl-7-/(5-methyl-1,2,4-oxadiazol-3-yl)methyl/-1*H*-purine-2,6-dione (D), was about eightfold stronger than that of the corresponding N-1-methyl analog, 3,7-dihydro-1,3-dimethyl-7-/(5-methyl-1,2,4-oxadiazol-3-yl)methyl/-1*H*-purine-2,6-dione (E)(14). The naturally occurring methylxanthines are shown; theobromine, 3,7-dihydro-3,7-dimethyl-1*H*-purine-2,6-dione (F), theophylline, 3,7-dihydro-1,3-dimethyl-1*H*-purine-2,6-dione (G), and caffeine, 3,7-dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione (H).

Fig. 2

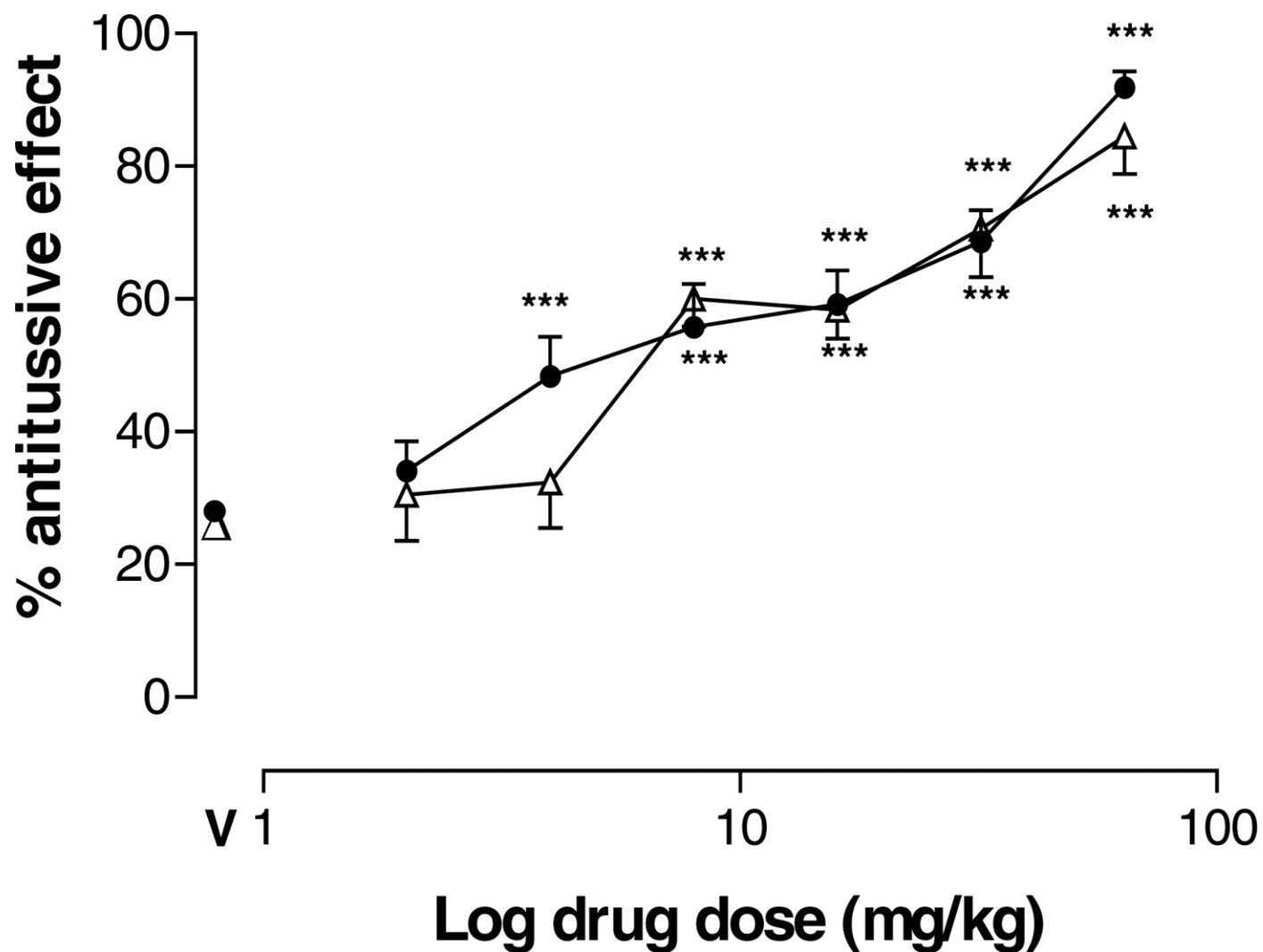


Figure 2. Dose–response antitussive effect of theobromine (●) and codeine (Δ) on citric acid-induced cough in conscious guinea pigs. Theobromine ($n=8$) and codeine ($n=6$) significantly inhibited citric acid-induced cough; theobromine at 4, 8, 16, 32, and 64 mg/kg, and codeine at 8, 16, 32, and 64 mg/kg when individually compared with the vehicle- (V) treated control groups. The antitussive effect with vehicle was 28.5% and 28% for theobromine and codeine, respectively. Logarithmic dose values shown represent mean \pm SEM, where *** denotes $P < 0.001$.

Fig. 3

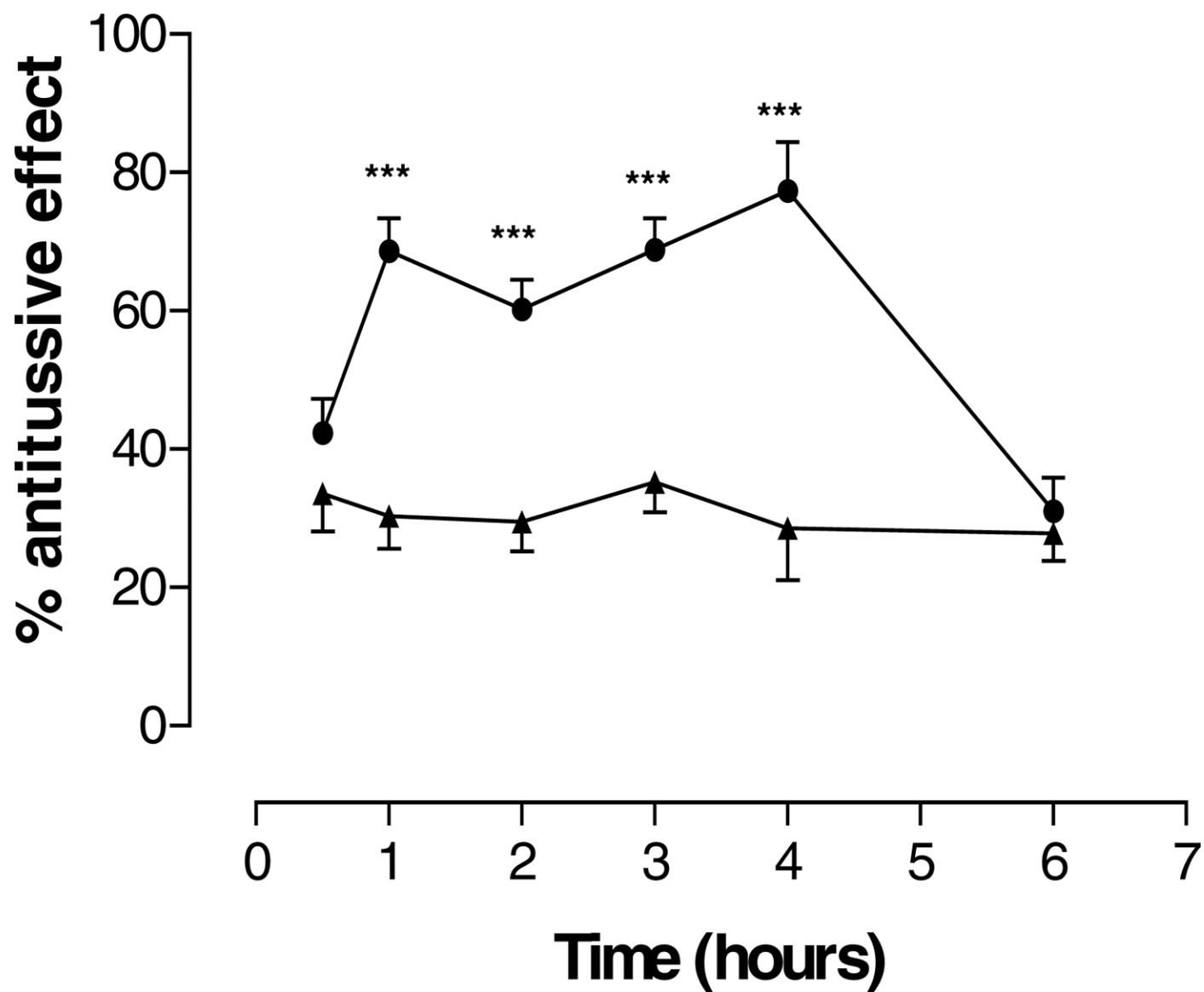


Figure 3. Time dependency of the antitussive effect of theobromine (●) on citric acid-induced cough in conscious guinea pigs. Theobromine at a dose of 32 mg/kg ($n=8$) significantly inhibited citric acid-induced cough after 1, 2, 3, and 4 h compared with vehicle control (▲). Values shown represent mean \pm SEM, where *** denotes $P < 0.001$.

Fig. 4

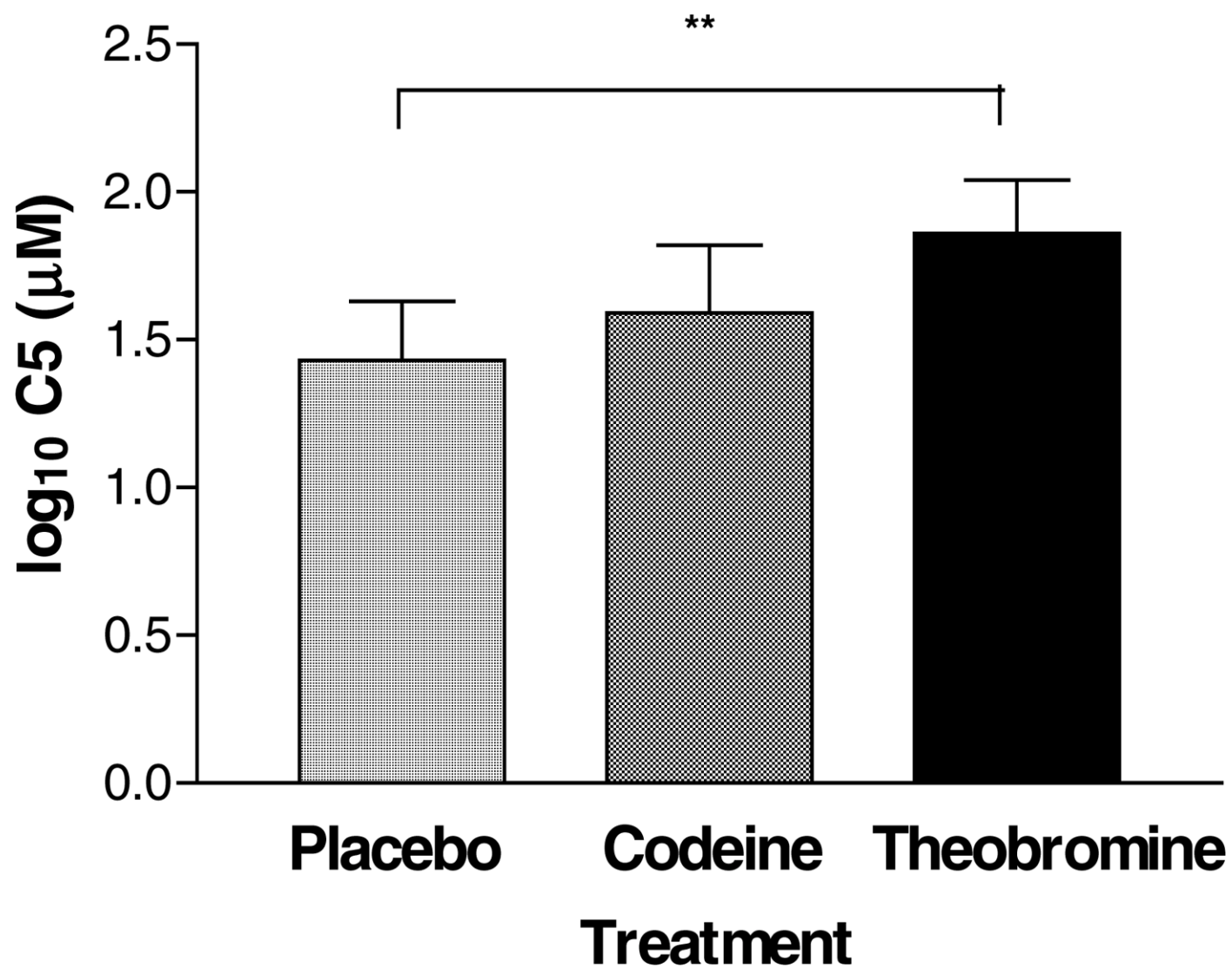


Figure 4. The effect of theobromine, codeine, and placebo on the capsaicin concentration required to induce five coughs (C5) in 10 healthy volunteers. Values shown represent mean \pm SEM, where ** denotes $P < 0.01$.

Fig. 5

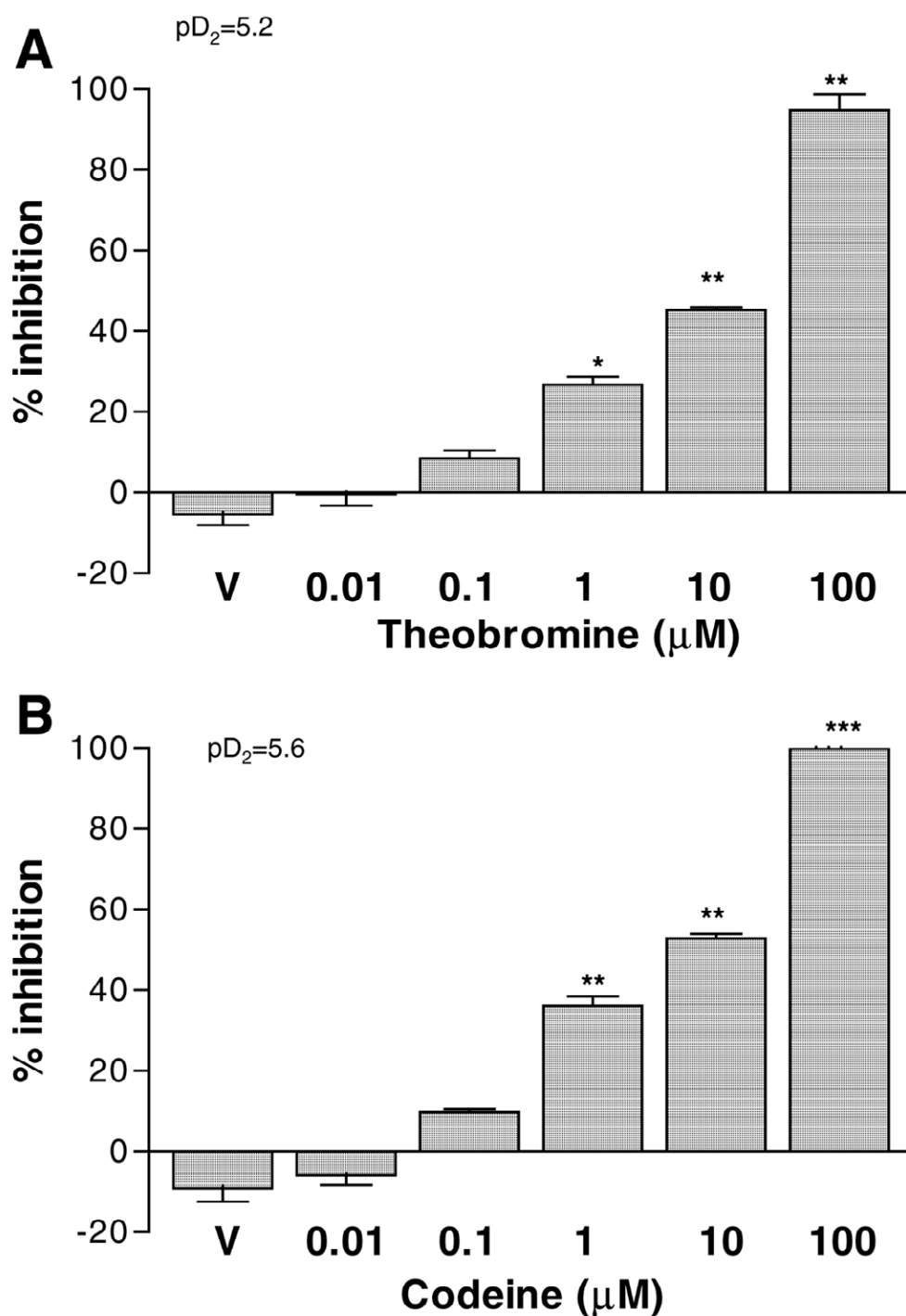


Figure 5. Inhibition of nerve depolarization by theobromine (**A**) and codeine (**B**). The inhibitory action of theobromine and codeine on capsaicin-induced nerve depolarization in isolated guinea pig vagus nerve preparations occurs in a concentration-dependent manner. Nerve depolarization responses were expressed as mV depolarization before (control response) and after drug additions and then expressed as a percentage change. V represents vehicle. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denote statistical significance compared with control responses in the same tissue before drug treatment. Values shown represent mean \pm SEM percentage change of $n = 4$ determinations in depolarizations compared with control response. The pD_2 values ($-\log_{10}$ of the EC_{50} defined as the concentration of drug required to elicit 50% of the maximal response) are shown.

Fig. 6

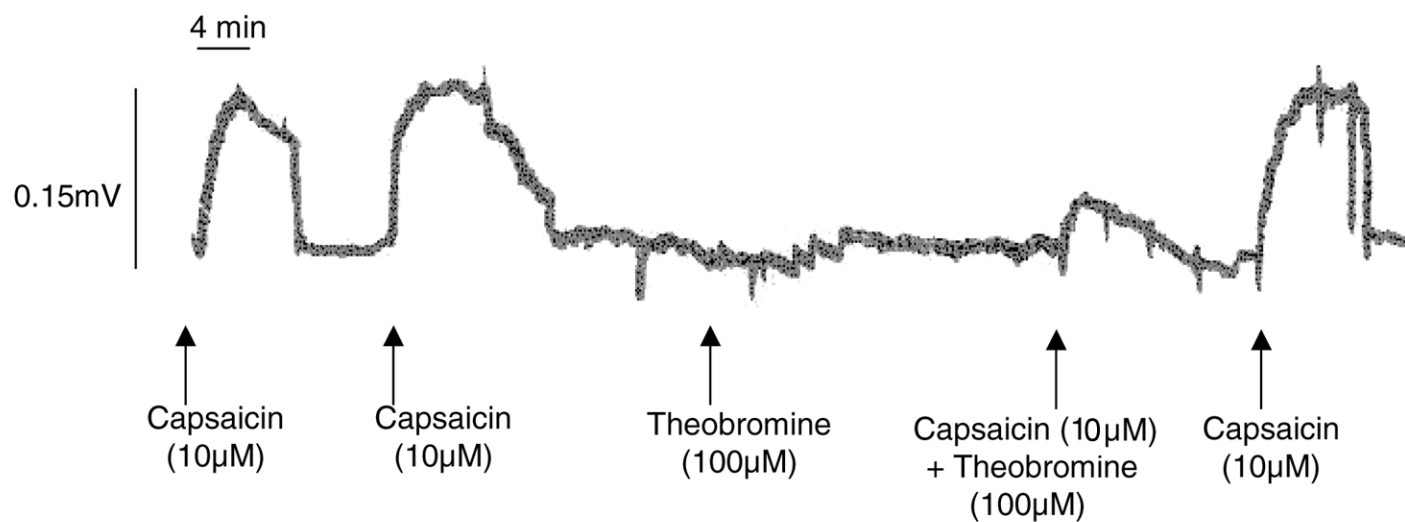


Figure 6. Theophylline mediated inhibition on capsaicin-induced nerve depolarization of isolated human vagus nerve. Tracing shows the inhibitory effect of theophylline on human vagus nerve depolarization induced by capsaicin.