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## PANCURONIUM MASKS THE PREJUNCTIONAL MUSCARINIC AUTORECEPTOR IN GUINEA PIG TRACHEAL SMOOTH MUSCLE

J.E. Nagtegaal<sup>||</sup>, H.L.M. Siero<sup>||</sup>, R. Dirksen\*, J.-W.J. Lammers<sup>‡</sup>, J.F. Rodrigues de Miranda<sup>||</sup> and  
F.G.M. Russel<sup>||</sup>

<sup>||</sup>Department of Pharmacology and \*Institute for Anaesthesiology, Faculty of Medical Sciences,  
University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, and <sup>‡</sup>Dept. of Lung Diseases,  
University of Utrecht, P.O. Box 80082, 3508 TB Utrecht, The Netherlands

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### Summary

The effect of pancuronium pretreatment on the function of the prejunctional muscarinic receptor in guinea-pig trachea was studied by using electrical field stimulation (EFS). The effects of cumulative doses of the muscarinic M<sub>2</sub> receptor antagonist gallamine were investigated in tracheal smooth muscle strips from guinea-pigs after addition of pancuronium *in vitro* and in strips from guinea-pigs which had been pretreated with doses of pancuronium that caused 100% neuromuscular blockade. The results of both types of experiments were compared to those of control groups of the same size. In all strips a dose response curve with cumulative doses of methacholine was made before EFS was switched on. No differences were found between the mean pD<sub>2</sub> value and slope of the concentration-response curves of untreated guinea-pigs and animals treated with anaesthetics and pancuronium. The animals showed variable responses to pancuronium. The bath concentration of pancuronium which decreased the EFS-induced contraction to half the original value varied between 14 - 61 µM. The intravenous dose necessary to paralyze the muscles, varied among the different guinea-pigs from 0.017 - 0.085 mg·kg<sup>-1</sup>. The EFS-induced contraction for the concentration range of gallamine 0.32 µM - 0.32 mM was found to differ significantly between the strips treated with pancuronium in the organ bath and their control group. For the guinea-pigs anaesthetized and pretreated with pancuronium a significant difference with control was observed at gallamine concentrations ranging from 0.032 - 0.32 mM. These results show that pancuronium, added to the organ bath as well as administered intravenously to the guinea-pig, masked the inhibitory muscarinic receptor.

**Key Words:** pancuronium, gallamine, muscarinic autoreceptor, inhibitory muscarinic receptor, tracheal smooth muscle, guinea-pig trachea

Prejunctional muscarinic autoreceptors have been demonstrated in functional and release experiments in different species like cats (1), rats (2), guinea-pigs (3-7), dogs (8) and man (4).

Corresponding author: Dr. F.G.M. Russel, Department of Pharmacology, Faculty of Medical Sciences, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands, Phone: +31 80 61 36 91, FAX: +31 80 61 42 14, E-mail: F.Russel@farm.kun.nl

The prejunctional autoreceptor has been characterized with selective muscarinic antagonists, and appeared to be an  $M_2$ -like receptor, which differs in the guinea-pig from the cardiac type  $M_2$  receptor in its relatively high affinity for hexahydrosiladiphenidol (6).

Prejunctional muscarinic autoreceptors in human airways are of special interest, as they might play a role in the etiology of asthma (9). The first evidence for the presence and functionality of prejunctional muscarinic autoreceptors in human airways came from a study in which pilocarpine and gallamine were used as selective agents to demonstrate the prejunctional autoreceptor in electrical field stimulation (EFS) experiments (4). Although one may question the usefulness of pilocarpine as a selective agonist in this approach (5), the possibility of a role of an inhibitory muscarinic receptor in human airways is important enough to perform further investigation.

In a subsequent study at our laboratory, with human bronchial smooth muscle obtained during lung surgery, however, no evidence was found for the presence of the prejunctional inhibitory muscarinic receptor with selective  $M_2$  antagonists (10). We therefore evaluated whether the specific conditions of lung surgery and in particular the various anaesthetic and neuromuscular blocking drugs used, may have caused the neuronal  $M_2$  receptor response to vanish. Specific attention was drawn to pancuronium, as pilot experiments had excluded the possible influence of halothane inhalation and intubation.

This study uses two different experimental approaches. In the *in vitro* experiments it is shown that incubation with pancuronium in the organ bath can cause the effect of gallamine on the inhibitory muscarinic receptor to vanish. In the second experimental approach clinical conditions were mimicked. Doses of pancuronium which caused complete muscle relaxation were administered intravenously. Tracheal smooth muscle strips of the animals did not show any effect of gallamine on the inhibitory muscarinic receptors.

### Methods

#### *Animals*

Healthy Dunkin-Hartley guinea-pigs of either sex (450-800 g, Harlan C.P.B., Zeist, The Netherlands) were used for the experiments. Pre-experimental conditions were standardized, including manufactured food and tap-water ad libitum, housing conditions, 12-h light-dark cycles with white light on at 08.00 a.m. and an environmental temperature of 21°C. The experiments were also performed at an environmental temperature of 21°C. Each guinea-pig was studied only once.

#### *Anaesthesia*

Anaesthesia was induced by inhalation of halothane in a mixture of air:oxygen (1:1). Once the animal stopped responding to external stimulation, the trachea was intubated. Anaesthesia was maintained with inhalation of halothane by a semi-open electrical-valve-controlled small-animal ventilator connected to the endotracheal cannula (Abbocath™ 14 gauge i.v. catheter), which was used for artificial normoventilation at a constant fresh gas flow of 0.5 ml·min<sup>-1</sup>·g<sup>-1</sup> body weight. For experiments under the same conditions in rodents of comparative weights it was found that such a minute volume resulted in a Pa<sub>CO<sub>2</sub></sub> of 4.5 - 5.0 kPa (11).

The left jugular vein was cannulated to allow the administration of drugs. The left tibialis anterior muscle tendon was freed, sectioned near its attachment and connected to a Ft03C Grass



force-displacement transducer. Twitch contractions were elicited by supramaximal stimulation of the proximally cut sciatic nerve on the same side. The (rectangular block) single stimuli were delivered by a Grass S11 stimulator at a rate of 0.1 Hz with a duration of 0.2 ms. The resting tension was adjusted to 15 g. Rectal temperatures were maintained between 37 and 38°C. The quantitated responses were recorded on a Thermal Array multichannel recorder together with the heart rate, which was recorded from the carotid artery.

#### *In vitro pancuronium experiments*

Guinea-pigs were killed by cervical dislocation, without the administration of anaesthetics. The trachea was dissected free from connective tissue and tracheal smooth muscle strips (two cartilage rings, strip length: 10 mm × 3 mm) were prepared in Krebs-Henseleit (KH) buffer. The composition of KH (in mM) was as follows: NaCl, 118.4; KCl, 4.7; MgSO<sub>4</sub>, 0.6; KH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25.0; CaCl<sub>2</sub>, 2.5; glucose, 11.1, continuously gassed with a mixture of 5% CO<sub>2</sub> and 95% O<sub>2</sub> in order to maintain oxygen tension and a pH of 7.4 at 37°C. Immediately after preparation, the strips were mounted between two platinum electrodes (above and under the preparation) in 10 ml organ baths (KH, 37°C) under isotonic recording with a preload of 0.25 g. After a 60-min equilibration period with five intermediate changes of buffer solution, a steady baseline was reached.

Cumulative doses of methacholine were added to the organ bath and the contraction of the tracheal strip was recorded. After a washing out period of 60 min, electrical field stimulation (EFS) was started, delivered by a Grass S48 stimulator. Every 100 s the strip was stimulated by a train of pulses with the following parameters: frequency 32 Hz; Grass reading voltage 70 V (voltage between the electrodes 15 V); pulse duration 0.5 ms and train duration 10 s. After a minimum period of 60 min, EFS-induced contraction height had become constant and pancuronium was added to the bath in cumulative doses until the contraction had decreased to 50% of its initial value (range of final bath concentration: 14 - 61 μM). When the EFS-induced contraction had been stable for at least 15 min, cumulative doses of gallamine were added with steps of 0.5 logarithmic units. Doses of gallamine were given when the EFS-induced contraction was stabilized. In the absence of a change the next dose was added after 15 min. When EFS-induced contraction height had decreased to 25% of its value before adding gallamine, EFS was stopped and the antagonist was washed out during 60 min. Thereafter, maximal contraction was determined with methacholine and maximal relaxation with isoproterenol (bath concentration: 10 μM) and EDTA (bath concentration: 3.2 mM). Four smooth muscle strips of four guinea-pigs were handled this way.

#### *In vivo pancuronium experiments*

Aliquots of pancuronium were administered intravenously at 5 min intervals until the twitch response of the tibialis anterior muscle was depressed completely. In pilot studies it had been assessed that once twitch contractions were completely depressed, this effect would last for at least 60 min. After a period of 45 min of complete muscle relaxation, the anaesthetized guinea-pig was removed from the ventilator and killed directly by cervical dislocation. Subsequently the trachea was freed, the smooth muscle strips were prepared and suspended in the organ baths, where they were treated according to the method described above. After the washing period and the addition of cumulative doses of methacholine, methacholine was washed out and EFS was started. When EFS-induced contraction had been stable for at least 15 min, cumulative doses gallamine were added with steps of 0.5 logarithmic units. Doses of gallamine were given when the EFS-induced contraction response was stabilized. In the absence of a change the next dose was added after 15 min. Each of the six smooth muscle strips of the six guinea-pigs was treated this way.

### *Control experiments*

For both types of experiments a control group was studied. Each was composed of animals of a similar weight and housed under identical conditions as the test group. These guinea-pigs were not anaesthetized, but were killed immediately by cervical dislocation. To verify whether gallamine enhances the EFS-induced contraction response during 50% postsynaptic receptor blockade, four control experiments were done with the M<sub>3</sub>-selective antagonist 4-DAMP. Similar to the *in vitro* pancuronium experiments, 4-DAMP was added to the bath until contraction was reduced to 50% of its initial value (final bath concentration 0.8 - 1.0 nM), whereafter the effect of increasing gallamine concentrations was measured.

To characterize the EFS-induced contraction response, the sodium channel blocking agent tetrodotoxin (bath concentration: 1 µM), the muscarinic antagonist atropine (bath concentration: 1 µM) or the nicotinic ganglion blocking drug hexamethonium (bath concentration: 10 µM) were added to four different tracheal smooth muscle strips of four different guinea-pigs.

### *Drugs*

Gallamine triethiodide, tetrodotoxin and atropine sulphate were obtained from Sigma Chemical Co (St. Louis, MO, USA). Acetyl-β-methylcholine chloride (methacholine) was obtained from Janssen Chimica (Beerse, Belgium). Hexamethonium iodide was purchased from Fluka (Buchs, Switzerland), pancuronium bromide from Organon Teknika (Boxtel, The Netherlands), 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) from Research Biochemicals International (Natick, MA, USA) and isoproterenol from Lansberg (Uden, The Netherlands). Chemical agents were of analytical grade. All drugs for the *in vitro* studies were prepared daily in Krebs-Henseleit buffer. Pancuronium bromide used *in vivo* was dissolved in 0.9% saline.

### *Data analysis and statistics*

The relative EFS-induced contraction height was defined as the measured EFS-induced contraction height (H<sub>m</sub>) divided by EFS-induced contraction height before any addition of antagonist (H<sub>c</sub>). Methacholine pD<sub>2</sub> values were calculated by using GraphPAD version 4.03 (GraphPAD Software Inc., San Diego, CA, U.S.A.). All values are given as mean ± SEM. At each bath concentration of gallamine the values of the relative EFS-induced contraction height with and without addition or administration of pancuronium were compared using Student's t-test for independent samples. In all experiments, *n* represents the number of guinea-pigs studied. Differences were considered to be statistically significant when *P* < 0.05.

## Results

### *Response to methacholine*

The mean pD<sub>2</sub> for methacholine in the airway smooth muscle strips from anaesthetized guinea-pigs treated with pancuronium was 5.68 ± 0.11 (*n* = 6), with a slope of the concentration-response curve of 0.65 ± 0.02 (*n* = 6, significantly different from 1). The mean pD<sub>2</sub> for methacholine in the strips of the guinea-pigs that were not anaesthetized and not treated with pancuronium was 5.46 ± 0.15 (*n* = 10), with a slope of 0.70 ± 0.04 (*n* = 10, significantly different from 1). The difference between the two groups was not significant. Although methacholine is susceptible to the action of cholinesterase, the lack of difference between the groups was not affected by inclusion of physostigmine (results not shown). In the *in vitro* experiments, the concentration-response curve to methacholine was shifted 0.58 ± 0.13 (*n* = 4) logarithmic units to the right in presence of 14 µM pancuronium, whereas the maximal response remained unaffected.

### Characterization of the EFS-induced contraction

EFS-induced contractions were blocked completely by atropine at a bath concentration of 1  $\mu\text{M}$ . Hexamethonium had no effect at a bath concentration up to 10  $\mu\text{M}$ , whereas tetrodotoxin completely inhibited the EFS-induced contractions at a bath concentration of 1  $\mu\text{M}$ .

### Influence of *in vitro* incubation with pancuronium on the effect of gallamine

In the absence of pancuronium, gallamine (3.2 nM - 1 mM) causes an increase of the EFS-induced contraction of the smooth muscle strips (Fig. 1). In the presence of pancuronium in the bath (14 - 61  $\mu\text{M}$ ) the increase of the EFS-induced contraction by gallamine is vanished. The differences are significant at the following bath concentrations: 0.32  $\mu\text{M}$  ( $P < 0.05$ ), 1 - 32  $\mu\text{M}$  (all:  $P < 0.01$ ), 0.1 mM ( $P < 0.02$ ), 0.32 mM ( $P < 0.05$ ). When contraction was reduced to half its initial value with 4-DAMP (0.8 - 1.0 nM), relative contraction responses significantly larger than 1 were observed at gallamine concentrations ranging from 0.032 - 0.32 mM, with the highest value of  $1.72 \pm 0.13$  at 0.32 mM. The results indicate that under these conditions gallamine still potentiates the EFS-induced contraction. Pancuronium alone produced an effect similar to gallamine on EFS-induced contraction. Maximal potentiation of the relative contraction response by  $1.20 \pm 0.06$  ( $P < 0.05$ ,  $n = 8$ ) was observed at a bath concentration of 1  $\mu\text{M}$ .

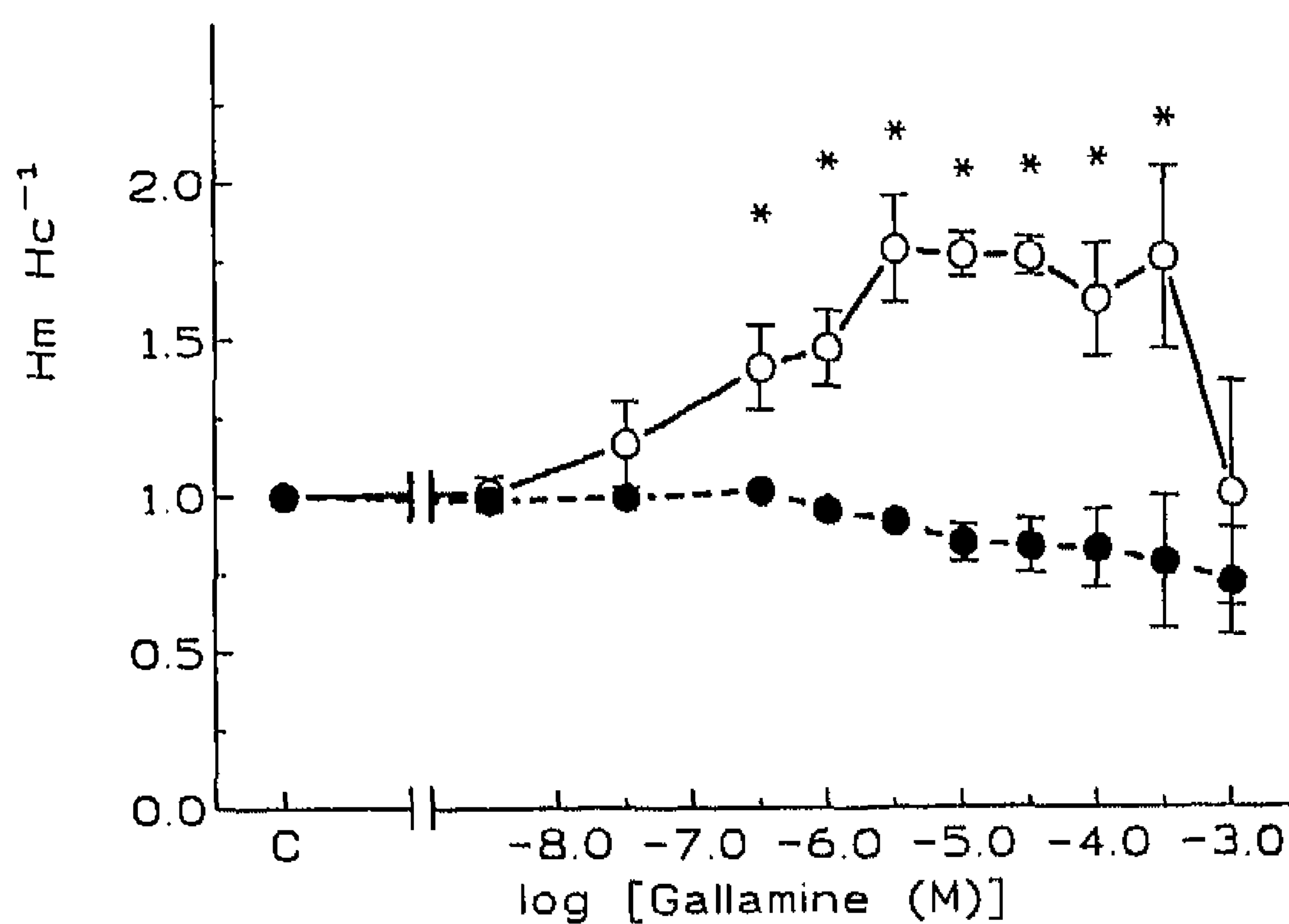


Fig. 1

The effect of gallamine on EFS induced contraction of smooth muscle strips of guinea-pig trachea in presence of pancuronium, at a concentration which caused 50% inhibition of the EFS induced contraction. The points represent the relative contraction ( $H_m H_c^{-1}$ , y-axis) at a certain gallamine concentration (bath concentration in M, logarithmic scale, x-axis).  $H_m$  = measured EFS induced contraction height;  $H_c$  = control EFS induced contraction height i.e. before addition of gallamine; C = control value: without addition of gallamine. Closed symbols correspond to strips in presence of pancuronium in the organ bath, open symbols indicate strips of the control group. Both groups consisted of four smooth muscle strips of four different animals (\* $P < 0.05$ )

### Influence of *in vivo* administration of pancuronium on the effect of gallamine

The intravenous dose of pancuronium that caused complete muscle relaxation in the animals varied within the range of 0.017 - 0.085  $\text{mg}\cdot\text{kg}^{-1}$ . From a representative experiment the effect as a function of time after intravenous administration of pancuronium is shown in Fig. 2. In the



untreated animals, gallamine (3.2 nM - 1 mM) causes an increase of the EFS-induced contraction of airway smooth muscle strips (Fig. 3). In the pancuronium treated animals increase of the EFS-induced contraction by gallamine is vanished. The differences significant at the following bath concentrations: 32  $\mu$ M ( $P < 0.02$ ), 0.1 mM ( $P < 0.01$ ), 0.32 mM ( $P < 0.02$ ).

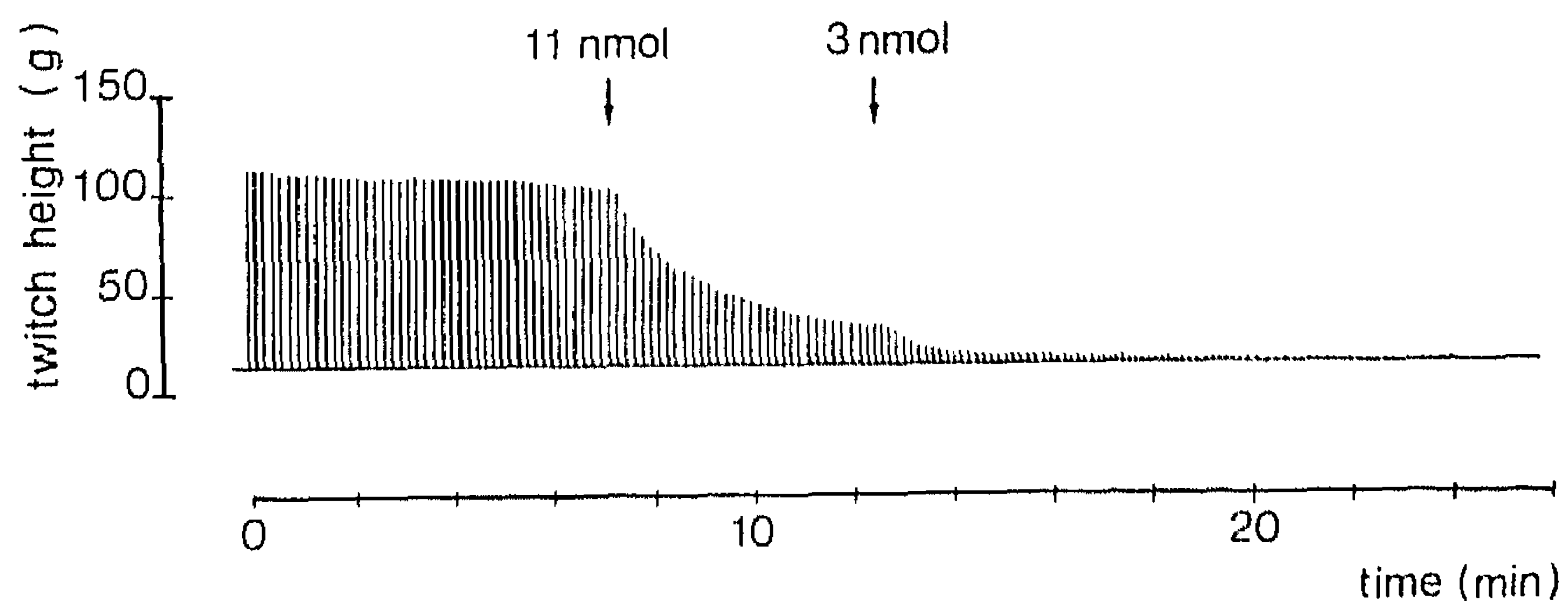


Fig. 2

A trace illustrating the effect of pancuronium, given intravenously, on the twitch of the tibialis anterior muscle in an anaesthetized guinea-pig

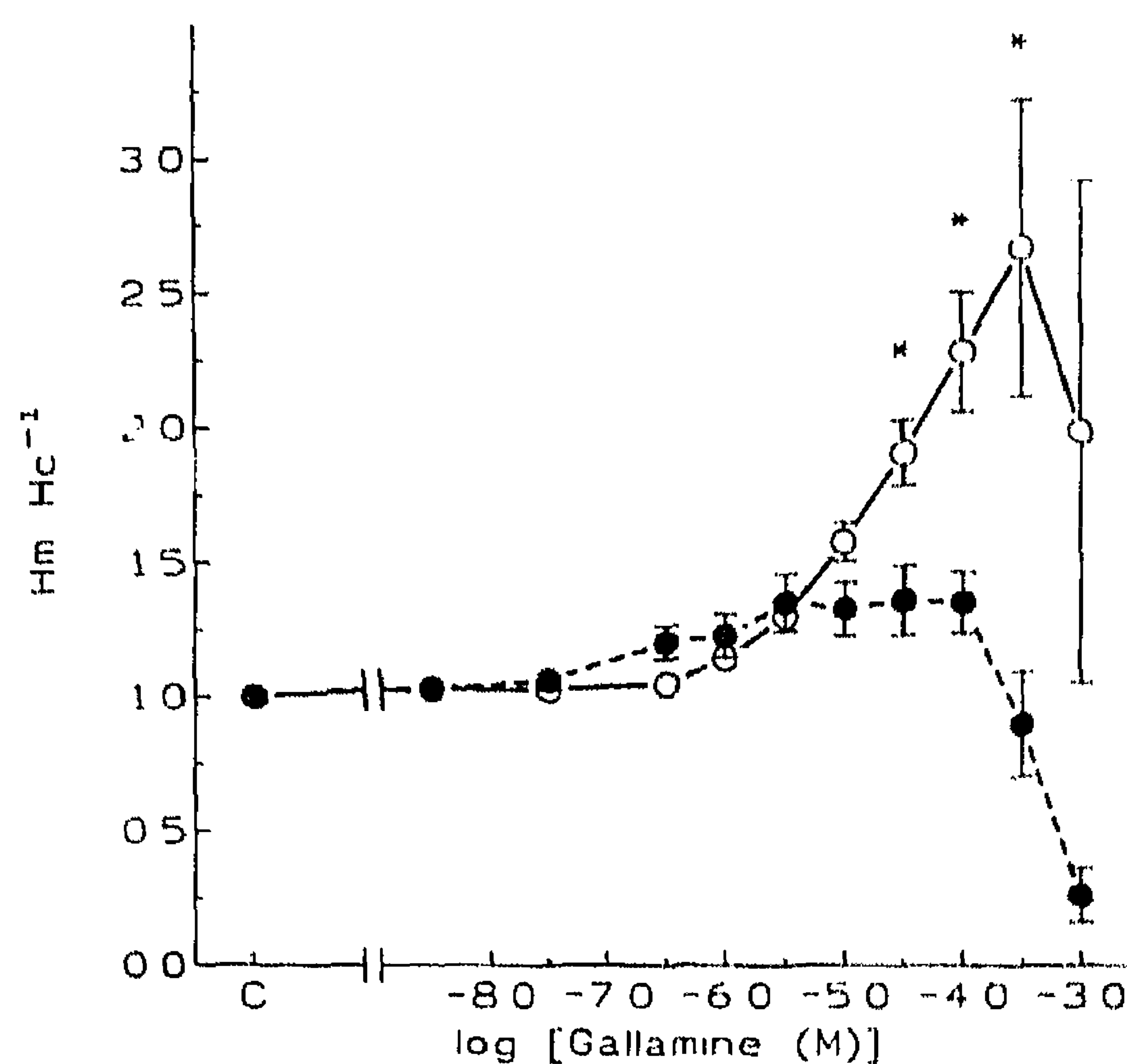


Fig. 3

The effect of gallamine on the EFS induced contraction of tracheal smooth muscle strips from guinea-pigs pretreated with pancuronium intravenously. The points represent the relative contraction ( $H_m/H_c$ , y-axis) in the presence of gallamine (bath concentration in M, logarithmic scale, x-axis).  $H_m$  = measured EFS induced contraction height;  $H_c$  = control EFS induced contraction height i.e. before addition of gallamine; C = control value: without addition of gallamine. Closed symbols correspond to strips from guinea-pigs which were pretreated with pancuronium intravenously, open symbols indicate strips of the controls. Both groups consisted of six smooth muscle strips of six different animals (\* $P < 0.05$ )

### Discussion

In the experimental setup we have chosen two approaches. In the *in vitro* approach we applied a concentration of pancuronium which caused a 50% blockade of the postsynaptic muscarinic receptor. Based on a 10- to 100-fold greater antagonist potency of pancuronium for muscarinic  $M_2$  receptors in the heart compared to its potency on  $M_3$  receptors in ileal smooth muscle (12), we assumed that at the concentration used an almost complete blockade of the prejunctional muscarinic receptors occurs. The concentration of pancuronium in the organ bath is well defined, but the clinical circumstances are mimicked incompletely. In the *in vivo* approach, which most clearly mimics the clinical situation, a critical dose of pancuronium could be chosen which causes complete relaxation of the skeletal muscle. The concentration of pancuronium in the receptor compartment after preparation of the smooth muscle strip, suspension in the organ bath, equilibration and washings and methacholine dose response curves are less defined. EFS-induced contraction of smooth muscle strips was blocked for at least 90% by the sodium channel blocking agent tetrodotoxin. Thus, EFS-induced response was completely nerve-stimulated and did not result from a direct activation of neural varicosities or endplates without nerve conduction (13). Since EFS-induced contractions were completely blocked by the muscarinic antagonist atropine (bath concentration: 1  $\mu$ M) and not influenced by the nicotinic ganglion blocking drug hexamethonium (bath concentration: 10  $\mu$ M), it may be concluded that the EFS-induced contraction with parameters as described, is mainly due to postganglionic, cholinergic stimulation. However, guinea-pig tracheal smooth muscles receive a dual innervation from both the parasympathetic and sympathetic nervous system and evidence exist for cross talk between the two neuronal systems (14-16).

Blockade of prejunctional inhibitory muscarinic receptors by selective antagonists will result in EFS experiments in an increased release of acetylcholine, which can be measured as an increased EFS-induced contraction, as is demonstrated in the control experiments with gallamine (the 'gallamine effect') in Figs. 1 and 3. A decrease in EFS-induced contraction can be explained by a postjunctional blockade of muscarinic receptors located on the smooth muscle. This postjunctional blockade occurs at high gallamine concentrations. The present study shows that the response of the inhibitory muscarinic receptor in guinea-pig trachea vanishes when pancuronium was used during anaesthesia. These data and their clinical relevance are discussed further.

Pancuronium is known for its nondepolarizing neuromuscular blocking action. Besides this action an effect on muscarinic receptors (17) and a cocaine-like action (18) are described. This latter autonomic effect of pancuronium might explain the observed suppression of the 'gallamine effect' as the parasympathetic nerve endings in the guinea-pig trachea contain inhibiting muscarinic as well as inhibiting adrenergic receptors (15). A more direct explanation for the observed suppression of the gallamine effect by pancuronium is its effect on the inhibitory muscarinic receptor.

Pancuronium has been classified as an antagonist of cardiac  $M_2$  muscarinic receptors, based on findings in the pithed rat and organ bath experiments with rat isolated atria preparation. Binding of [ $^3$ H] quinuclidinyl benzilate to cardiac  $M_2$  muscarinic receptors of the rat was inhibited by classical muscarinic antagonists but also by nicotinic blocking agents and by inhibitors of acetylcholinesterase (19). Gallamine and pancuronium were among the most potent of the examined agents, they showed no agonist activity but, like atropine, completely antagonized muscarinic receptor-mediated inhibition of cyclic AMP formation. From *in vivo* experiments it was concluded that pancuronium and gallamine are antagonists for prejunctional  $M_2$  and



postjunctional  $M_3$  muscarinic receptors in the guinea-pig (12). Both agents potentiated vagally-induced bronchoconstriction. In our *in vivo* guinea-pig experiments, no measurable amounts (<0.08 ng/mg tissue) of pancuronium were found in homogenates of smooth muscle strips following the washing procedure, as determined with HPLC (results not shown). Although one could speculate about a selective accumulation of pancuronium in the receptor compartment, these findings show that a direct blockade of prejunctional muscarinic receptors seems unlikely under these conditions.

Minette and Barnes (4) investigated the  $M_2$  receptor in human bronchus with the agonist pilocarpine but they could not find significant evidence for the  $M_2$  receptor with gallamine. The authors suggested that a residual concentration of atropine, which was used preoperatively, may be the cause of not reaching the level of significance with gallamine. The authors did not mention whether pancuronium or other muscle relaxants were used during anaesthesia. We could not find the prejunctional inhibitory muscarinic receptor in human bronchial smooth muscle with a series of selective antagonists either. One of the explanations suggested for the inability to identify inhibitory muscarinic receptors in human bronchus was that the anaesthetic drugs used during surgery may interfere with the experiments (10). Because of its antimuscarinic effects, pancuronium was considered to be of special interest (20,21).

Our study clearly demonstrates the influence of clinically relevant doses of pancuronium on the action of gallamine, although the mechanism underlying this phenomenon remains unknown. Clinical experiments by Minette et al. (9) indicated that prejunctional inhibitory muscarinic receptors may protect against parasympathetic stimuli in healthy subjects, whereas these receptors seemed to be dysfunctional in mild asthmatics. When pancuronium causes the inhibitory muscarinic receptor response to vanish *in vivo* and this receptor plays a protective role, one may wonder why the use of pancuronium in lung surgery has not resulted in any bronchospasms or asthmatic attacks. It might be interesting to focus coming investigations on this item.

The inhibitory muscarinic receptor is significantly less functional in presence of pancuronium, either intravenously administered or added to smooth muscle strips in the bath. Based on these results we expect that the administration of pancuronium during lung surgery has caused the apparent absence of muscarinic autoreceptors in our earlier experiments on human bronchus tissue. It will be interesting to compare pancuronium clinically as well as in animal experiments with other muscle relaxing agents without muscarinic activity (e.g. norcuronium).

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