An in vitro model for studying neuromuscular transmission in the mouse pharynx

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Abstract

The muscles of the pharynx are controlled by networks of neurons under the control of specific regions in the brainstem, which have been fairly well studied. However, the transmission between these neurons and the pharyngeal muscles, at the motor end plates, is less well understood. Therefore an *in vitro* model for studies of neuromuscular transmission in pharyngeal muscle of the mouse was developed. Ring preparations from the inferior constrictor and the cricopharyngeus muscles were isolated and mounted for isometric force recording at physiological temperature. Preparations from the diaphragm and the soleus muscles were examined in parallel. The muscles were stimulated at supramaximal voltage with short tetani at 100 Hz. Following direct stimulation of the muscle fibers, using a longer pulse-duration, the rate of force development of the pharyngeal muscles was similar to that of the diaphragm and faster than that of the soleus muscle. By varying duration of the stimulation pulses, conditions where the nerve mediated activation contributed to a major extent of the contractile responses were identified. Gallamine completely inhibited the nerve-mediated responses. In separate experiments the dose dependence of gallamine inhibition was examined, showing similar sensitivity in the inferior pharyngeal constrictor compared to the diaphragm and soleus muscles. We conclude that reproducible contractile responses with identifiable nerve induced component can be obtained from mouse inferior pharyngeal constrictor. The pharyngeal muscles have contractile characteristics similar to those of the faster diaphragm. The sensitivity to the neuromuscular blocking agent gallamine of the inferior pharyngeal constrictor was in the same concentration range as that of the diaphragm and soleus muscles.

Introduction

Swallowing involves numerous interacting control mechanisms and is under the control of specific regions of the brain stem where the activity of ´pattern generators´ is modulated by higher brain centra and by sensory feedback. Studies of the neuronal architecture, the presence of neurotransmitters, and in vivo studies of nerve stimulation have revealed information about the central organization of swallowing (1). The location and tentative functions of several neuropeptides in the central nervous nuclei have also been described (2). The main functional target for the motor neurons from these central nuclei is the pharyngeal musculature active during swallowing. Characterization *in vitro* of muscles of the pharynx has previously been done (3, 4, 5, 6) on muscles from rabbits. However, the neuromuscular transmission in pharyngeal muscles has only been examined in two prior studies (7, 8) and to our knowledge studies on mice have not been presented.

The integrative physiological processes during swallowing are affected by a number of clinically important pharmaceutical compounds. One important example is the neuromuscular blocking agents. Their effects on the pharyngeal muscles have, until recently, been largely unknown (9). There are strong indications that partial neuromuscular block impairs airway protection as pulmonary aspiration has been reported after priming doses of neuromuscular blocking agents (10).

Our understanding of swallowing and its pathophysiology has been advanced by a number of clinical studies in man. However, there is a need for well defined animal and *in vitro* models for study of pharyngeal physiology, in order to specifically address mechanistic issues in the swallowing process. The causes for dysphagia are complex and multifactorial but further

information on specific cellular and genetic processes or novel models for development of therapies may be obtained by the use of transgenic mice. Although some transgenic mice strains, e.g. mice with an oculopharyngeal muscular dystrophy mutation, exhibit alterations in the structure of pharyngeal muscle, information on pharyngeal muscle function in such models or normal mice has not been presented (11, 12). This study presents an *in vitro* model for studying neuromuscular function in the pharyngeal muscles of the mouse. We have compared properties of these muscles with those of the diaphragm and soleus muscle and examined the effects of one neuromuscular blocking agent, gallamine.

Material and methods

Animals and preparations

Young adult female NMRI mice (n=20) weighing about 30 g were used (B&K Universal, Sollentuna, Sweden). The mice were killed by cervical dislocation and care was taken not to damage the pharyngeal area. The anterior abdominal wall was then opened in the midline and this incision was then extended into the thoracic wall. The anterior thoracic wall was cut open by incisions bilaterally in the thoracic cage through the ribs and clavicles. The lungs and the trachea with attached esophagus were identified and the esophagus was gently dissected from the prevertebral tissues. The dissection was then continued up into the cervical region and the lungs were removed. The preparation that now contained the trachea, larynx and base of the tongue anteriorly and the esophagus and pharynx posteriorly was cut as cranially as possible usually through the base of the tongue and the upper extreme of the pharynx. The preparation was placed in a glass container for further dissection. The trachea and larynx were cut open in the anterior midline. This incision was continued through the hyoid bone which was located in the preparation's extreme cranial anterior part. Thereby the posterior wall of the trachea as well as the vocal cords, epiglottis and posterior wall of the pharynx were exposed. A two mm wide ring was cut including the thyroid cartilage and the vocal folds. The upper extension of the ring was just above the vocal folds and the lower extension was between the thyroid and cricoid cartilages. Thus this preparation represented the inferior pharyngeal constrictor and was easily recognized with its characteristic median posterior raphe. This tissue specimen also included the vocal cords. A transsection was made immediately below the cricoid cartilage. Thereby a 1-2 mm wide ring including the cricopharyngeus muscle was obtained and was identified by the lack of a median raphe posteriorly.

The ring preparations of the inferior pharyngeal constrictor and the cricopharyngeus muscles contained some loose adhering tissue (including sectioned strap muscles, connective tissue, cartilage), which was preserved in order not to disturb the nerve and muscle tissue. In addition, the diaphragm was isolated keeping part of the rib cage and the fibrous central plate for muscle attachment. The soleus muscles and their tendons were dissected from each hind leg of the animal. The animal experiments were approved by the local animal ethics committee.

Mounting for force recording

The pharyngeal muscles, i.e., the inferior pharyngeal constrictor and the cricopharyngeus, were mounted as ring preparations. Loose loops of 6/0 silk thread were tied around the lateral extremes of the muscle. The loops were not tightened around the muscle fibers. The preparations were then transferred to a muscle holder in a 50 ml open organ bath. One of the loops was attached to a horizontal Perspex bar, while the other was connected to a Grass FT03 (Grass Medical Instruments, Quincy, MA) force transducer. One of the soleus muscles was mounted in the holder and attached to the force transducer with silk thread tied around the tendons. An approximately 1 mm wide segment of parallel muscle fibers was dissected from the diaphragm and attached to the holder and force transducer with silk thread tied at the fibrous central part and around the rib cage cartilage region. We thus examined four muscle preparations simultaneously, the inferior pharyngeal constrictor, the cricopharyngeus, the diaphragm and the soleus. The force transducers were mounted on adjustable stands and the muscle length could be adjusted for each muscle individually.

The experiments were performed at 37^{o}C in a modified Krebs solution of the following composition (in mM): 4.74 KCl, 25.2 $NaHCO_2$, 1.19 $MgCl_2$, 1.19 KH_2PO_4, 11 glucose, 117.2

NaCl, 2.5 $CaCl_2$ gassed with 95% O2/5% CO_2 giving a pH of 7.4. The amplified signal from the force transducer was recorded, stored and analyzed using a computerized system (A/D instruments Power lab).

Stimulation protocols

The muscle holder for each muscle was equipped with platinum electrodes mounted in parallel on both sides of the preparations (the gap was about 5 mm). Electrical field stimulation was performed using Grass S48 stimulation unit at supramaximal voltage. In initial test experiments on the different muscles, we applied tetani (5 s duration, 100 Hz) with 0.5 ms pulses and increased the stimulation voltage from the level just eliciting a contraction. When the force did not increase further we considered this voltage level to be optimal and continued the stimulation at a voltage level about 20% above optimal. Since muscle size did not vary and since the mounting geometry was identical we kept these parameters throughout the study. Since the pharyngeal preparations were mounted as ring preparations a pure length tension curve could not be recorded. The preparations were mounted at a low passive preload tension for recording of isometric contractions. It was confirmed that this length was close to optimal by stimulation at different lengths of the muscle. Two series of experiments were performed: (1) activation with varied pulse duration with and without a maximal dose of gallamine and (2) activation using constant pulse duration with varied concentrations of the gallamine.

Effects of pulse duration

The primary purpose of these experiments was to define stimulation conditions where the nerve-mediated stimulation could be separated from the direct stimulation (direct muscle activation) of the muscle. A 100 Hz tetanic stimulation (giving a fused tetanus) was applied

with varying duration of the pulses (0.01-0.5 ms). The duration of the tetanic stimulation train was 5 s. This time was found to give strong and reproducible contractions. Muscles were activated at 2-minute intervals allowing recovery between tetani, and giving reproducible contractions. After mounting the preparations two to three activations with 0.5 ms stimulus duration were performed to ensure that the contractions were reproducible and the preparations were not damaged during the preparation procedure. The muscles were activated at 2-minute intervals with 5 seconds stimulation trains at 100 Hz as described above. The pulse duration was gradually increased (0.01, 0.02, 0.03, 0.05, 0.085, 0.1, 0.3, 0.5 ms). The shortest durations were assumed to contain a relatively large nerve mediated component, whereas the longest pulse duration elicited a relatively large direct stimulation of the muscle. The force at the end of the tetanus (after 5 s) was recorded.

After recording this initial pulse duration-response curve the preparations were allowed to rest for 30 minutes. During this period gallamine was introduced. A second pulse duration-response curve was recorded in the presence of the drug. Gallamin 0.2 mM (a maximal dose) was used in these experiments to determine the contribution of nerve stimulation at the different pulse durations.

Concentration-response relationships

The purpose of this series of experiments was to investigate the concentration-effect relationship of gallamine on nerve-mediated responses. Stimulation conditions with a large nerve-mediated component were used. Muscle specimens were mounted and initially stimulated maximally at 0.5 ms duration as described for the protocol above. A short pulse duration where the relative contribution of the nerve activation is maximal was then chosen. In the experiments described above, we found that stimulation durations giving about 50% of

the maximal response contained a large nerve-induced component in all muscles (cf. Results, Figure 2). The pulse duration was therefore adjusted in the range 0.02-0.04 ms to obtain a response which was approximately 50% of that at 0.5 ms. Stimulations with this duration were then repeated at 3-minute intervals. The dose of the drugs was increased in steps at 15-minute intervals. We evaluated force during the initial maximal peak and after 5 s. Forces were related to the maximal response in the absence of drug. In this more extensive stimulation protocol we found that force at the end of the experiment (after about 20 tetani) was lower also in the absence of gallamine. To evaluate the loss of force during the repeated 5 s tetanic stimulations, control experiments were therefore performed where muscles were stimulated in the absence of drug.

Statistics

All values are given as mean ± SEM. All curve fitting and statistical analysis was performed using SigmaPlot and SigmaStat for Windows (SPSS Inc., Erkrath, Germany).

Results

Nerve induced responses

Figures 1 shows comparative recordings of 5 s tetani from the pharyngeal (inferior pharyngeal constrictor and the cricopharyngeus) and the two control (soleus and diaphragm) muscle preparations *in vitro*. At a maximal pulse duration (0.5 ms), rapid and sustained contractions were recorded from all muscles. For comparison, the tetanic contraction of the soleus muscles developed slower compared to that of the diaphragm muscles (half-time for tension development, $t_{½}$: soleus 71±4 ms, n=9, diaphragm 43±6 ms, n=8). In these respects the pharyngeal muscle was faster than the soleus and similar to the diaphragm ($t_{½}$ of tension development inferior pharyngeal constrictor: 40±9 ms, n=8, cricopharyngeus 37±6 ms, n=6). The diaphragm and the pharyngeal muscles has significantly shorter $t_{½}$, compared with the soleus ($p<0.05$, Bonferroni corrected Student's t-test).

The contractions elicited with longer stimulus durations (0.5 ms) were little affected by gallamine, showing that this stimulation duration involved almost complete direct stimulation of the muscles, overriding the nerve-muscle synapses. In contrast, when the preparations were activated using shorter pulse duration the contractions were weaker and significantly inhibited by gallamine.

Effects of varied pulse duration and of gallamine

Figure 2 shows summarized data for the plateau force of contractions at different pulse durations. The right panels of the Figure show per cent inhibition by gallamine at different pulse durations. In the interval of pulse duration between 0.01 to 0.03 ms the nerve-mediated component amounted to the large part of the contraction. For these pulse durations, force prior

to addition of gallamine was about 50% of maximal or less. The unaltered maximal force of the muscles (stimulation at 0.5 ms, left panels of Fig 2) shows that the force generating ability of the muscles was not affected during the experiments. In control experiment for determining the dose-dependence (Fig 3, discussed below), muscles were repeatedly stimulated (each 3 minutes) at a stimulus duration giving half maximal force (i.e. corresponding to the mid points in Fig 2). The force of these contractions at intermediate pulse duration after 12 stimuli (approximately corresponding the time between the two contractions at intermediate duration in Fig 2) were decreased by ±0% in the soleus, 25±9 in the diaphragm and 29±7% in the inferior pharyngeal muscle. Since this stimulation protocol would be more intense than that used for Figure 2, it would give an upper limit for the decay of force at intermediate stimulation duration. Using these values the control values (open circles in left panels of Fig 2) would be lowered by a maximum of 0, 25 and 29% relative to their current values in soleus, diaphragm and inferior pharyngeal constrictor, not reaching the values in the presence of gallamine. These considerations do thus not affect our main conclusion regarding the nerve component at the intermediate stimulation durations.

Dose-dependence for gallamine

To examine the concentration dependence of gallamine we used a condition where the nerve-mediated component was high. Each muscle preparation was therefore activated using a pulse duration giving 50% of the maximal force at 0.5 ms duration. The durations used were 0.031±0.014 ms for diaphragm, 0.084±0.015 ms for soleus, and 0.051±0.009 ms for the lower pharyngeal constrictor. These contractions were repeated every 3rd minute at increased concentrations of gallamine. In separate control preparations, repeated contractions were induced in the absence of the drug. The effect of gallamine occurred with a half-time of about 3 minutes and therefore we allowed 15 minutes at each concentration to achieve full effect.

During the course of these experiments the control preparations lost some force (force at end of experiment: diaphragm 68 ± 5, n=8; soleus: 100 ± 9, n=7; inferior pharyngeal constrictor 65 ± 9, n= 7. Values in percent of initial force). The cricopharyngeus preparations lost tension (about 50%) during the repeated nerve-stimulation protocol above, which did not enable the dose dependence for gallamine to be determined with accuracy in this preparation. The analysis of the dose-dependence of gallamine was therefore restricted to the inferior pharyngeal constrictor in comparison with diaphragm and soleus. Control contractions at 0.5 ms stimulus pulse duration (stimulating the muscle directly) were performed prior to and after the repeated contractions at short pulse duration. The force difference between these control contractions was only marginal showing that the force decrease in the control preparations during repeated stimulation was due to a gradual failure of the nerve induced responses. We therefore corrected for this, by subtracting the force decrease in the controls at each time-point during the experiments where gallamine was added. From these data we constructed gallamine dose-response relations for the different muscles, as shown in Figure 3. Each experiment was analyzed to determine the EC_{50} for gallamine inhibition. The EC_{50} values were approximately 50 μM and similar for the three different muscle preparations (diaphragm: 59±14; soleus: 31±10; inferior pharyngeal constrictor 53±28 μM, n=4-5).

Discussion

We describe an *in vitro* model for analysis of muscle function and neuromuscular transmission in pharyngeal muscle of the mouse. Using this model we show that the rate of force development of the inferior pharyngeal constrictor and the cricopharyngeus was comparatively fast, similar to that of the diaphragm and faster than that of the soleus. The cholinergic activation of the inferior pharyngeal constrictor was inhibited by the neuromuscular blocking agent gallamine in a concentration interval similar to that of the diaphragm and the soleus striated muscles.

The *in vitro* experiments of the striated muscle in the pharynx introduce several technical challenges. The experiments were performed at 37ºC, which can be a situation where the muscle energy supply, in relation to metabolism, might be limited. However, we considered it important to examine the nerve-muscle transmission and contraction at a physiological temperature. The experiments were performed in maximally oxygenated solutions and we did not observe any major decrease in the maximal force of the muscle during the experiments. The muscles cannot be attached via tendons and we adopted a technique with ring preparations. This does not enable the length-force relations of the muscles to be determined in detail but this mounting procedure minimizes the possibility of damage to the muscle cells.

Our initial aim was to define an *in vitro* model where nerve-induced responses could be characterized from pharyngeal muscle in comparison to the diaphragm and soleus. We chose the two latter muscles since the fast muscle of the diaphragm is a previously well-studied preparation with regard to its neuromuscular transmission. The soleus muscle represents a slower skeletal muscle type. Since we could not isolate nerves to the pharyngeal muscle, we

chose to stimulate the whole muscle preparations *in vitro* and thereby activating nerve and nerve-endings included in the preparations. To allow direct comparisons, similar procedures were used for the diaphragm and soleus. In order to discriminate the nerve-evoked contractions from the direct muscle cell stimulation we identified stimulation conditions where the nerve component could be identified. Both the pharyngeal muscles had significant nerve-induced contractions. However, the relationship between pulse duration and force (Fig. 2) was shifted towards higher pulse durations, the gallamine sensitive component was smaller, and the nerve-induced contractions were less well maintained during repeated tetanic stimulations in the cricopharyngeus muscle. This might suggest a less well developed nerve component in this muscle. Mu and Sanders (6) using a Sihler´s stain technique from adult autopsy specimens have demonstrated that the intramuscular nerve distribution is very rich in the inferior pharyngeal constrictor and relatively sparse in the cricopharyngeus muscle. This could explain why our technique in the mouse was less efficient in evoking prominent and reproducible nerve-mediated contractions in the latter muscle while the technique was more reliable in the inferior pharyngeal constrictor.

It could be argued that examination of nerve muscle preparations ideally should be performed by isolation and stimulation of the respective nerves *in vivo* followed by recording of the muscle tension. However, as the *rami pharyngii* are localized immediately adjacent to the skull base, such a preparation is not easily achieved. Further, *in vivo* muscle contraction force of the pharynx has to be recorded using intraluminal manometry. We therefore aimed to design a reproducible and stable technique for examination of the pharyngeal nerve-muscle preparation *in vitro*. This requires, however, that nerve-mediated stimulation and direct muscle stimulation was correctly separated.

It has been shown (5), using adenosine triphosphatase staining that the inferior pharyngeal constrictor of dog is rich in type 2 fibers. We find that the rate of force development in tetanic stimulation was comparatively fast in the pharyngeal muscle, which suggests that the fibre type composition, also in the mouse, is of the fast type. This could reflect that this muscle is participating in rapid contractions during swallowing, and not primarily engaged in tonic sphincter-like contractions. We do not have corresponding information from the mouse cricopharyngeus, but in man this muscle has been shown to be a predominantly slow muscle with high content of type I fibers (6).

We find that the sensitivity to gallamine (EC_{50} ~50 µM) was similar to that described for isolated phrenic nerve diaphragm preparations (13, 14). Thus, the preparation used in the present study allows for an isolation of the nerve mediated component and an examination of the pharmacological sensitivity of the neuromuscular junction, although the nerve is not isolated and specifically activated.

It has been shown in man, using video manometric techniques, that pharyngeal muscles have a different sensitivity to partial neuromuscular blockade. After administration of a clinically used neuromuscular blocking agent (vecoronium) to human volunteers it was found that the inferior pharyngeal constrictor was less inhibited compared to the cricopharyngeus muscle, which behaved like the peripheral striated muscle during partial neuromuscular block (15, 16). However, our results in the mouse show that the inferior pharyngeal constrictor has a similar sensitivity compared the diaphragm and slow skeletal muscles.

In the present study we have characterized a model of isolated mouse pharyngeal muscle and examined effects of a neuromuscular blocking agent. At present novel mouse models are

introduced, which mimic neurodegenerative disease or muscle dysfunction associated with swallowing disorders in man (e.g. 11, 12, 17). In this context, the *in vitro*model as introduced here would be of interest to examine the neuromuscular aspects of the swallowing process.

There has been a recent interest for neuromuscular electrical stimulation (NMES) of the pharynx as a treatment modality for patients with pharyngeal dysfunction after stroke (18, 19, 20, 21). Such muscle stimulation protocols typically uses 3 volt and up to 25 mA 80 Hz alternating current. Our study has shown that at least in an *in vitro* model electrical nerve-mediated stimulation results in considerable muscle contraction in the inferior pharyngeal constrictor. By using various stimulation durations it would be possible to tune the stimulation parameters for activation of the pharynx via its nerves or directly of the muscle.

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Legends

Figure 1. Original recordings of force of tetanic contractions (supramaximal voltage, 5 seconds train duration, frequency 100 Hz) in muscle preparations from the diaphragm (Panel A), the soleus (Panel B), the inferior pharyngeal constrictor (Panel C), and the cricopharyngeus (Panel D), stimulated with a maximal (0.5 ms) and a intermediary (0.03-0.05) ms pulse duration in the absence and in the presence of 0.2 mM gallamine. Force values are expressed relative to maximal responses obtained at 0.5 ms pulse duration.

Figure 2. Relation between pulse duration and tetanic force in muscle preparations from the diaphragm (Panel A, n=6), the soleus (Panel C, n=4), the inferior pharyngeal constrictor (Panel E, n=9) and the cricopharyngeus (Panel G, n=6). The muscles were stimulated with supramaximal voltage and 5 seconds train duration at 100 Hz with. In the left panels (A, C, E, and G), the open circles show data obtained in the absence of gallamine and the filled circles show data on the presence of 0.2 mM gallamine. The right panels (B, D, F, and H) show the per cent inhibition at the different pulse duration for the three muscle preparations (B: diaphragm, D: soleus, F: inferior pharyngeal constrictor, H: cricopharyngeus), respectively.

Fig 3. Dose response relationship for gallamine in muscle preparations from the diaphragm (Panel A), the soleus (Panel B), and the inferior pharyngeal constrictor (Panel C). For each muscle preparation the pulse duration giving 50% of force obtained at a long pulse duration of 0.5 ms was determined. The muscles were then repeatedly stimulated using the short pulse duration and gallamine was added in increasing concentrations. The extent of inhibition, after 15 minutes at each dose, was expressed relative to the initial force in the absence of gallamine. In control experiments the extent of force decay during repeated stimulations in the absence of gallamine was determined and subtracted from the data in the presence of gallamine n=4-5.

Fig 1

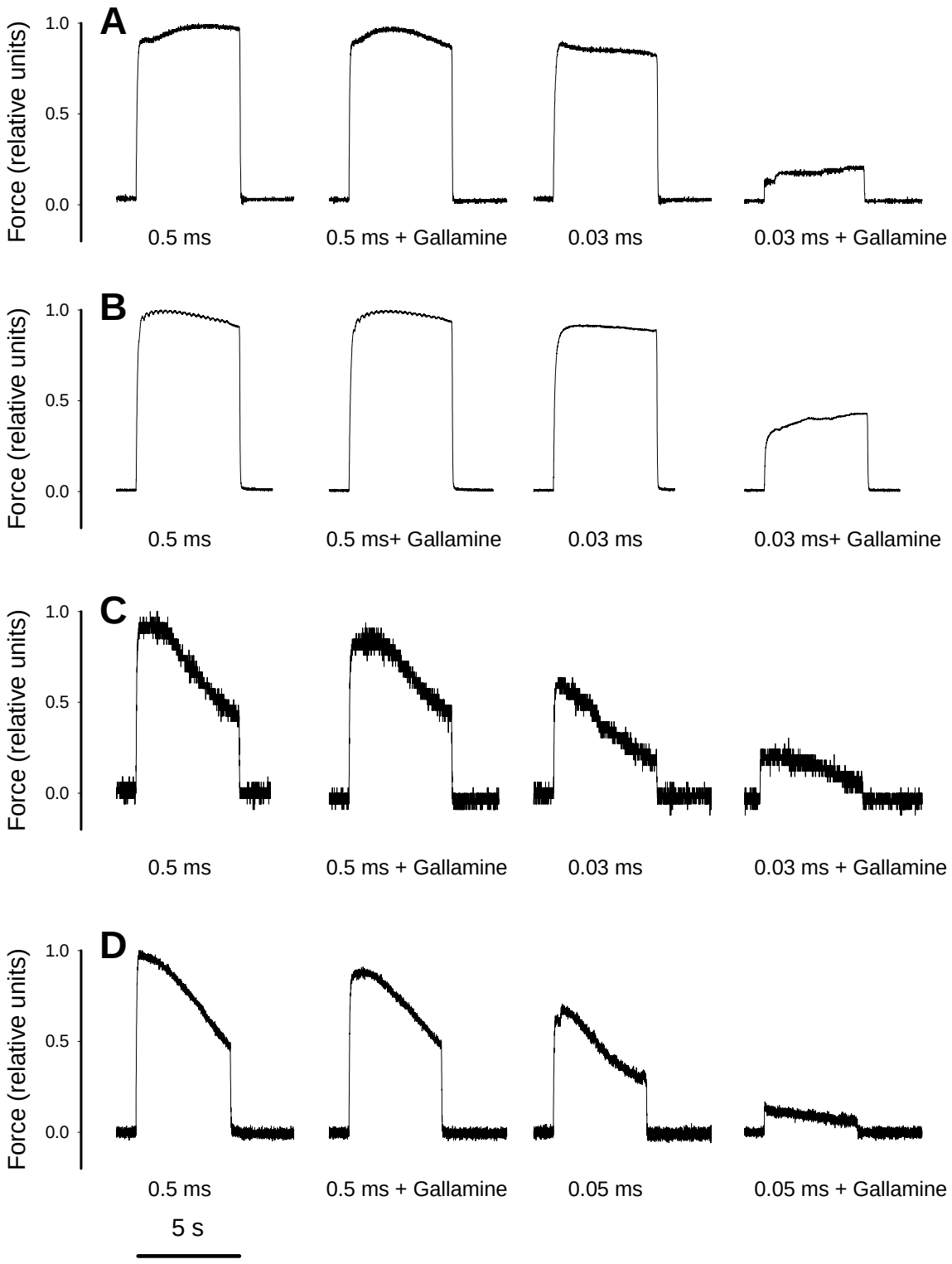

A
B
C
D
Force (relative units)
1.0
0.5
0.0
0.5 ms
0.5 ms + Gallamine
0.03 ms
0.03 ms + Gallamine
0.5 ms+ Gallamine
0.03 ms+ Gallamine
0.05 ms
0.05 ms + Gallamine
5 s

Fig 2

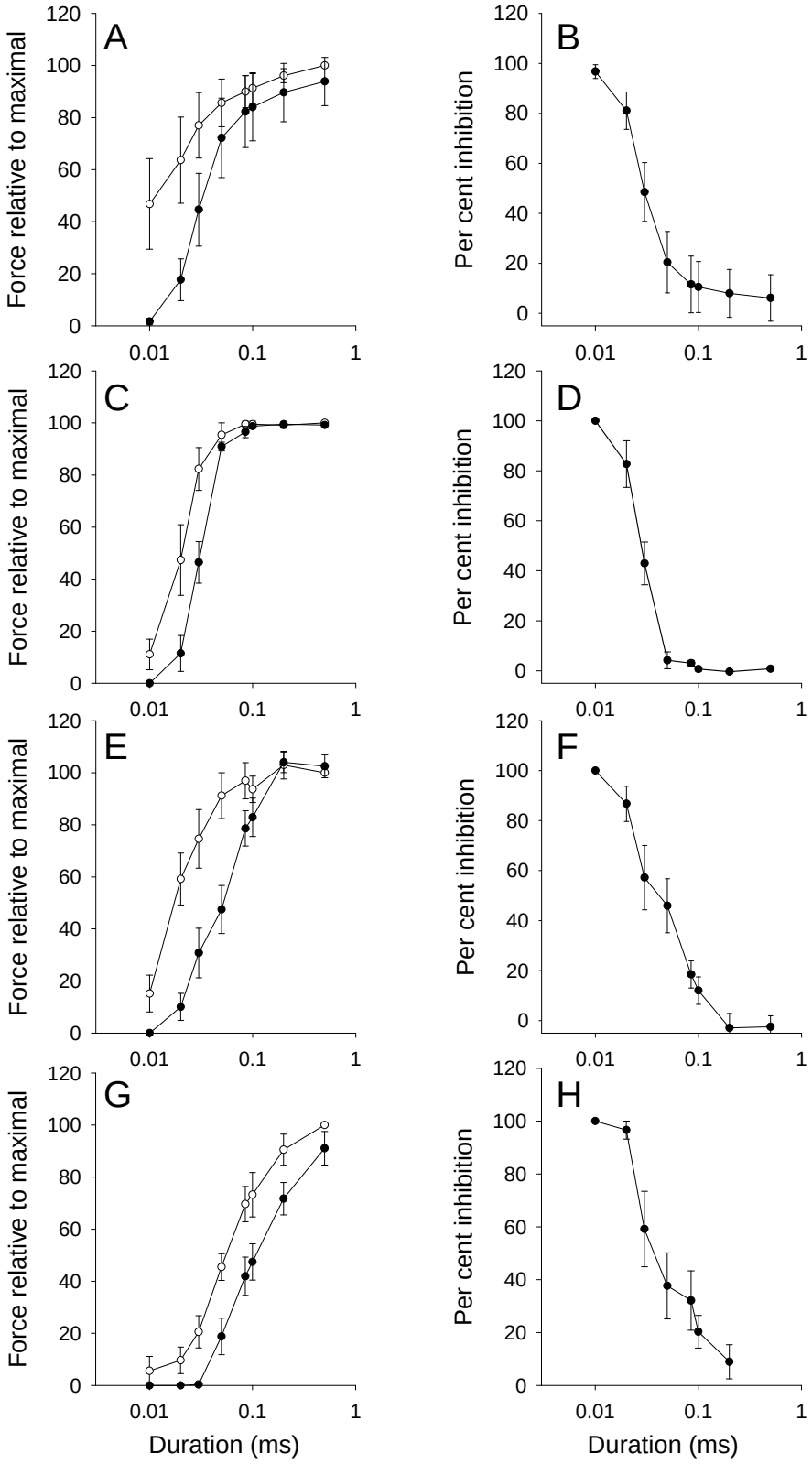
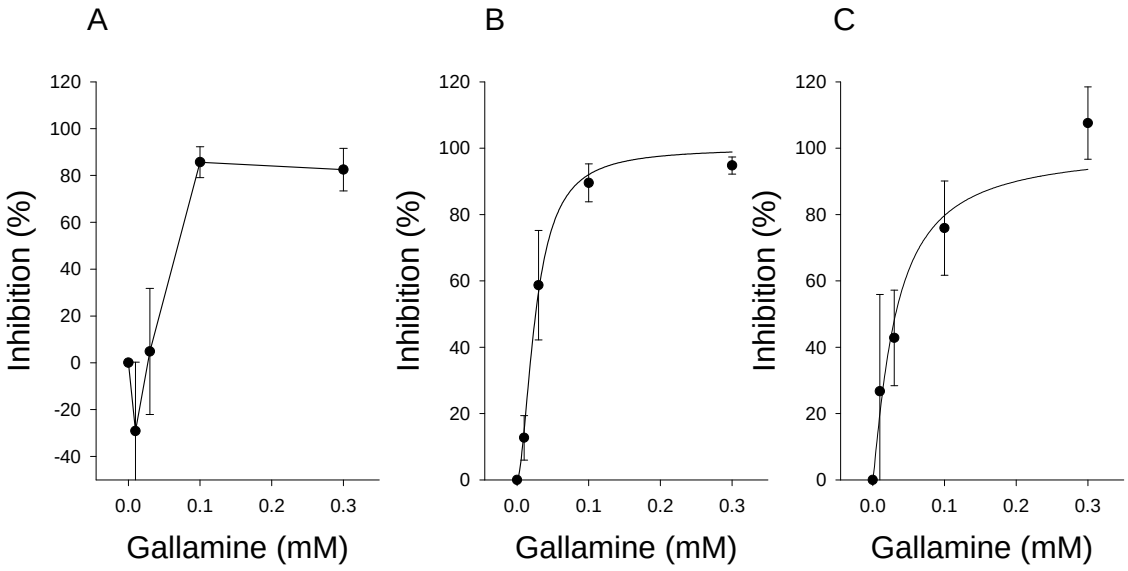

A
B
C
D
E
F
G
H
Force relative to maximal
Per cent inhibition
Duration (ms)
0
20
40
60
80
100
120
0.01
0.1
1

Fig 3


A
B
C
Inhibition (%)
Gallamine (mM)
120
100
80
60
40
20
0
-20
-40
0.0
0.1
0.2
0.3