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Force-frequency relationships of *in vivo* human and *in vitro* rat diaphragm using paired stimuli

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ABSTRACT: Supramaximal stimuli, with time intervals of 100 ms (10 Hz) to 10 ms (100 Hz), were delivered in pairs to the phrenic nerves, bilaterally, in five seated normal subjects, while transdiaphragmatic pressure swings (Pdi,s) were recorded at relaxed end-expiratory lung volume with airways closed.

In fresh diaphragms, Pdi,s increased between 10–20 Hz and reached a plateau between 20–30 Hz. Diaphragmatic fatigue decreased Pdi,s at all frequencies. Pdi,s was assumed to be the sum of two successive responses (T1+T2), T1 being constant at any frequency and equal to a single twitch, T2 being obtained by subtraction. We found that T2 amplitude, which was significantly reduced after fatigue, was fully returned to normal after 15 min rest at high, not at low, stimulation frequencies. The ratio of T2 at 10 Hz over 100 Hz ($T2_{10/100}$) thus decreased from 1.33±0.05 before fatigue to 0.97±0.12 after fatigue, and to 0.81±0.06 after 15 min rest.

Similar results were obtained in isolated rat diaphragmatic strips stimulated and fatigued *in vitro*, from which we found a highly linear relationship ($r=0.94$, $p<0.001$) between the ratio of $T2_{10/100}$ and that of tetanic force at 10 Hz over 100 Hz ($P_{10/100}$).

We conclude that phrenic nerve paired twitches provide similar information when obtained from phrenic tetanic stimulation in terms of diaphragmatic contractility, and the decrease in $T2_{10/100}$ ratio indicates diaphragm low frequency fatigue.

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Force-frequency curves contain much information about skeletal muscle contractility and are particularly useful in determining the type and severity of muscle fatigue. Skeletal muscle fatigue is conventionally divided into high and low frequency fatigue. In the former, there is a selective loss of force at high stimulation frequencies and recovery from it is rapid (usually less than half an hour), while in the latter, the selective loss of force occurs at low stimulation frequencies and recovery is prolonged (usually greater than an hour) [1–3]. High and low frequency fatigue can be easily detected by calculating the ratio of force development at a low stimulation frequency to that at a high stimulation frequency. This ratio increases with high frequency fatigue, while it decreases with low frequency fatigue [1–8].

Force-frequency curves of the diaphragm have been reported in humans for unilateral transcutaneous phrenic nerve stimulation, using transdiaphragmatic pressure (Pdi) response as an index of force [4, 9]. However, the Pdi generated in response to bilateral phrenic nerve stimulation more accurately reflects the strength of diaphragmatic contractions [10, 11]. Furthermore, unless supramaximal stimulation is administered, data interpretation becomes difficult, because one is uncertain that stimulus intensity remains constant at different frequencies. Trains of supramaximal stimuli are also very painful; the procedures can barely be tolerated unilaterally and not at all bilaterally. Single supramaximal shocks, on the other hand, are more easily tolerated.

In order to determine whether Pdi-frequency curves could be obtained from twitches, rather than trains of stimuli, we varied the interval between bilateral transcutaneous supramaximal phrenic nerve paired shocks from 10 ms (100 Hz) to 100 ms (10 Hz). This proved feasible in normal human subjects and much easier to achieve than tetanic stimulation. We found that the ratio of the second twitch Pdi at 10 Hz expressed as a ratio of that at 100 Hz ($T2_{10/100}$) is a valuable index of low frequency diaphragmatic fatigue. Further studies on rat diaphragmatic strips *in vitro* supported this conclusion. Thus, this paper describes a method of measuring the Pdi-frequency relationships *in vivo* in human and how it is affected by low frequency fatigue. It is potentially applicable as a clinical test.

Methods

Human study

Subjects. Experiments were performed on five normal male volunteers, all of whom knew the purpose of the investigation and had previous experience of being subjects for study of respiratory mechanics.

Pressure measurements. Oesophageal pressure (Poes) and gastric pressure (Pga) were measured by the conventional oesophageal and gastric balloon-catheter systems, originally described by AGOSTONI and RHAN [12] and MILIC-EMILI *et al.* [13]. All signals were recorded on tape.

Phrenic nerve stimulation. In the present study, either single shocks or paired stimuli, with interpulse intervals ranging from 10 ms (100 Hz) to 100 ms (10 Hz), were delivered to both phrenic nerves simultaneously *via* two synchronized constant current stimulators (Teca SC6). Figure 1 is a schematic illustration of the methods employed to stimulate the phrenic nerves and record the elicited diaphragmatic muscle mass action potentials (M-waves) with surface electrodes. Briefly, an electrode pad was placed just below the clavicle, near the sternum on each side, to serve as anode. The hand-held cathodes were applied bilaterally at the phrenic motor point in the neck, at approximately the lower posterior edge of the sternocleidomastoid muscles. The exact site and orientation of the cathodes were carefully adjusted, in order to provide the "best" stimulation condition as judged by the size of the M-wave and minimal involvement of the brachial plexus. The stimulus current was then progressively increased, until further increase of the current elicited no additional increase in the size of the diaphragmatic M-waves. Thereafter, the stimulus current was further increased by approximately 20–30% and kept constant throughout the test to ensure supramaximal stimulation.

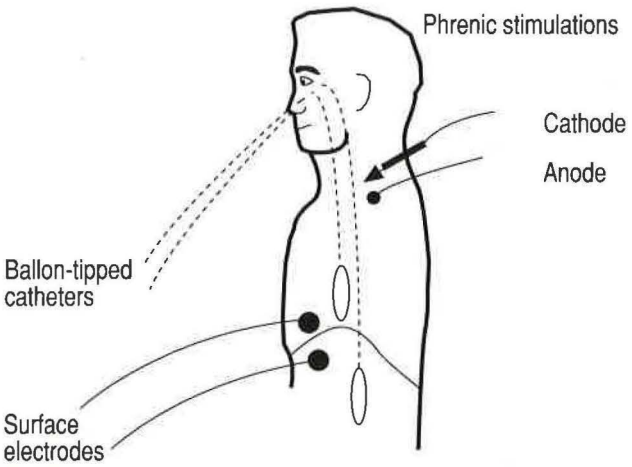

Fig. 1. – Schematic representation of the techniques to stimulate the phrenic nerves and to record the elicited diaphragmatic muscle mass action potentials (M-waves) by surface electrodes.

The diaphragmatic M-waves from both sides were recorded with bipolar surface electrodes. On each side one electrode was placed over the 6th or 7th intercostal space between the anterior axillary line and the mid-clavicular line, and the other over the adjacent costal margin. A more detailed description of the methods employed can be found elsewhere [14, 15].

Experimental protocol. The subjects were studied seated, with lower rib cage and abdomen tightly bound to minimize diaphragm shortening during elicited contractions. The binding consisted of inelastic tape wrapped around the subjects from the xiphoid to the iliac crest. The measurements were performed before and after the induction of diaphragm fatigue, and after 15 min rest post-fatigue. Diaphragm fatigue was induced by inspiring through an external resistance and generating a target Pdi of 60% of maximal voluntary Pdi, with a duty cycle of about 0.6 as described previously [15]. All measurements were made with the subjects relaxed against a closed glottis at end-expiratory lung volume. For each experimental condition at each of the selected frequencies (see results below), 5–8 stimuli to the phrenic nerves were administered and the elicited M-waves and pressure responses were recorded.

Animal study

Nine strips (length 2.05–2.43 cm, weight 0.05–0.09 g) of costal diaphragm were excised from five Sprague-Dawley rats, previously anaesthetized with pentobarbitol sodium (50 $mg \cdot kg^{-1}$ *i.p.*). The strips were mounted vertically between two platinum stimulating electrodes and immersed in Kreb's solution perfused with 95% O_2 and 5% CO_2 gas mixture at 25°C. This is not only the temperature optimal for maintaining stability of contractile properties [16], and of fatigue resistance [17] *in vitro*, but also the one with a twitch duration similar to that of humans *in vivo*. The Kreb's solution contained 118 NaCl, 4.5 KCl, 1.5 $MgSO_4$, 1.2 KH_2PO_4, 25.5 $NaHCO_3$, 3.2 $CaCl_2$, 5.6 glucose in mM, and 0.2% curare. The strips were stimulated directly and supramaximally with 0.2 ms square wave pulses, to contract isometrically with single and paired shocks as in the human study. In addition, the strips were also stimulated with four pulses and trains of pulses (duration 500 ms) from 10–100 Hz. Six strips were fatigued by supramaximal twitch stimulation (0.2 ms square wave pulses and 1.5 $pulses \cdot s^{-1}$) until the twitch force decreased to about 50% of the control value. After fatigue, and also following 15 and 30 min recovery, single twitches, paired stimuli and tetanic stimulation at 10 and 100 Hz were repeated. All measurements were made with the muscle strips held at the optimal length for single twitch stimulation [18]. The force output was recorded by a Kulite force transducer (Model: LOAD CELL BG-100 GRAMS) and expressed conventionally as Newtons per square centimetre ($N \cdot cm^{-2}$) [19].

Data management

For human studies, the pressure and M-wave signals recorded on tape were fed through a 12 bit analogue-to-digital convertor to a computer for data analysis. The sampling rate was set at 500 Hz for pressure signals and 200 Hz for M-wave signals. Pdi was obtained by digitally subtracting Poes from Pga. The electrical and pressure responses to the phrenic nerve shocks were isolated over an appropriate time window (usually 500 ms) and then averaged by the computer. At least five responses were averaged, at each frequency, under each condition. Peak amplitude of the averaged signals was measured relative to the immediately preceding baseline and referred to as Pdi,s. The force recorded in animal studies was directly fed to the computer through the analogue-to-digital convertor. Five single twitches were averaged in each condition. The analysis was the same as in the human study.

In this paper, a single response refers to the electrical or mechanical response to a single shock, *i.e.* an ordinary twitch, whereas a paired response refers to the response to paired stimuli. A paired response, in turn, is assumed to be the sum of two successive responses, the first of which is constant for a given condition and equal to the single response, the second response being the residual between the paired and the first responses. Based on this assumption, the second M-waves (M2) and the second twitches (T2) (Pdi in human study or tension in animal study) were obtained by digital subtraction on computer.

Statistical assessment was performed using the paired t-test, the analysis of variance (ANOVA), and standard linear regression techniques, as appropriate. The criterion for statistical significance was $p<0.05$. Values reported in the text and given in figures are mean±1SEM.

Results

Human study

Electrical responses. Representative M-wave responses from one subject at four different frequencies are shown in figure 2 (upper panel). The middle panel of figure 2 shows the corresponding second M-waves (M2) obtained by subtraction. The amplitude of M2 progressively decreased with increasing frequency (ANOVA $p<0.001$), so that at 100 Hz, it was 78% of the amplitude of a single M-wave. However, this change was not influenced by fatigue and recovery. The first M-wave (M1) was independent of frequency

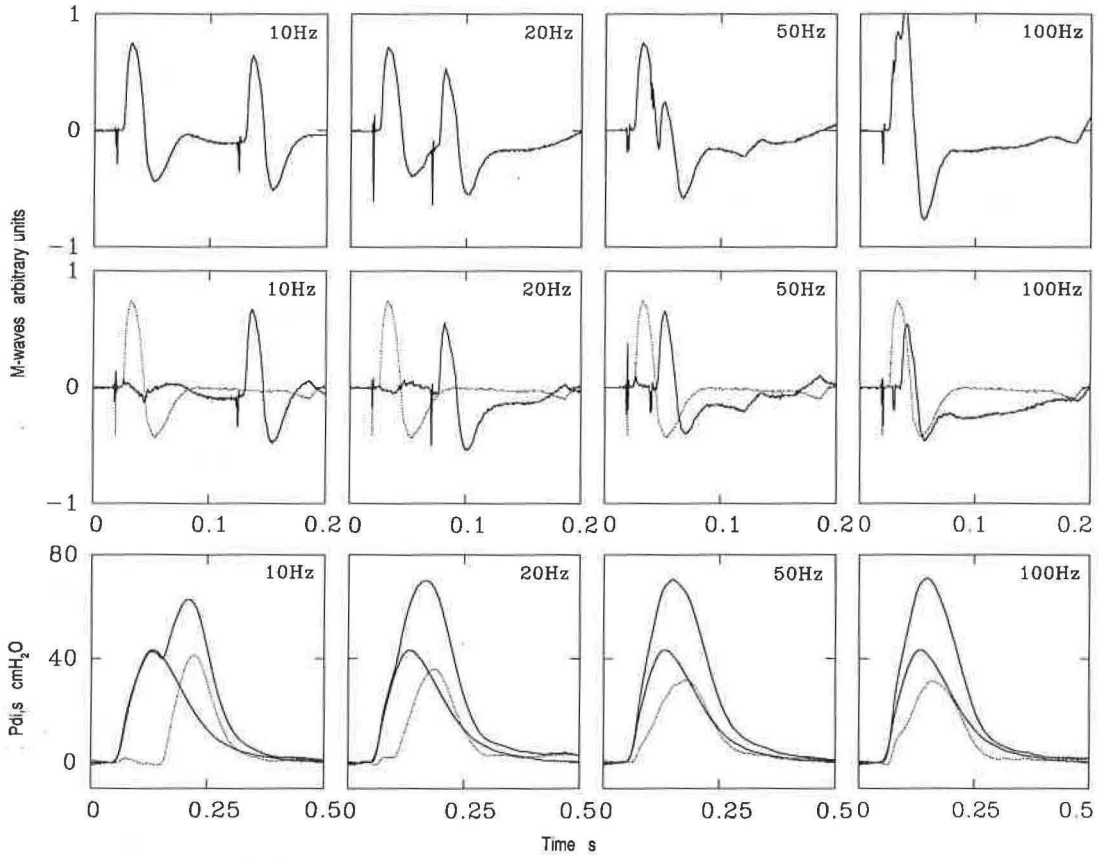

Fig. 2. – Upper panel: records of M-waves from left hemidiaphragm of one subject in response to paired shocks at four stimulation frequencies. Middle panel: records of the second M-waves (M2) obtained after subtraction at each corresponding frequency. An M-wave response to a single shock is also shown at each frequency (dotted tracings). Lower Panel: Representative transdiaphragmatic pressure (Pdi) responses to single shock (lower solid curve) and to paired shocks (upper solid curve) are shown for one subject. Each panel is superimposed by the second twitch response (T2) (dotted curve) obtained by subtraction at that frequency (see methods).

but significantly different as a result of fatigue and recovery compared to the fresh condition (ANOVA $p<0.001$). However, since the mean amplitudes of M1 in the three conditions were, in fact, close (fresh 98.6±0.7%; fatigue 105.6±1.6%; and recovery 93.6±1.6%) to that of a fresh single M-wave, the differences were considered probably to be not physiologically meaningful.

Mechanical responses. Examples of Pdi responses to paired stimuli at four tested frequencies are shown for one subject in the lower panel of figure 2. The first (T1) and second responses (T2) are also indicated. The summation of Pdi responses to each of the two successive stimuli is clearly seen at 10 Hz, but less clearly at 20 Hz and at higher frequencies. In three of the subjects, repeated determinations of Pdi,s- frequency relationships were made on 2–3 separate days, over a period of 1–20 weeks (fig. 3).

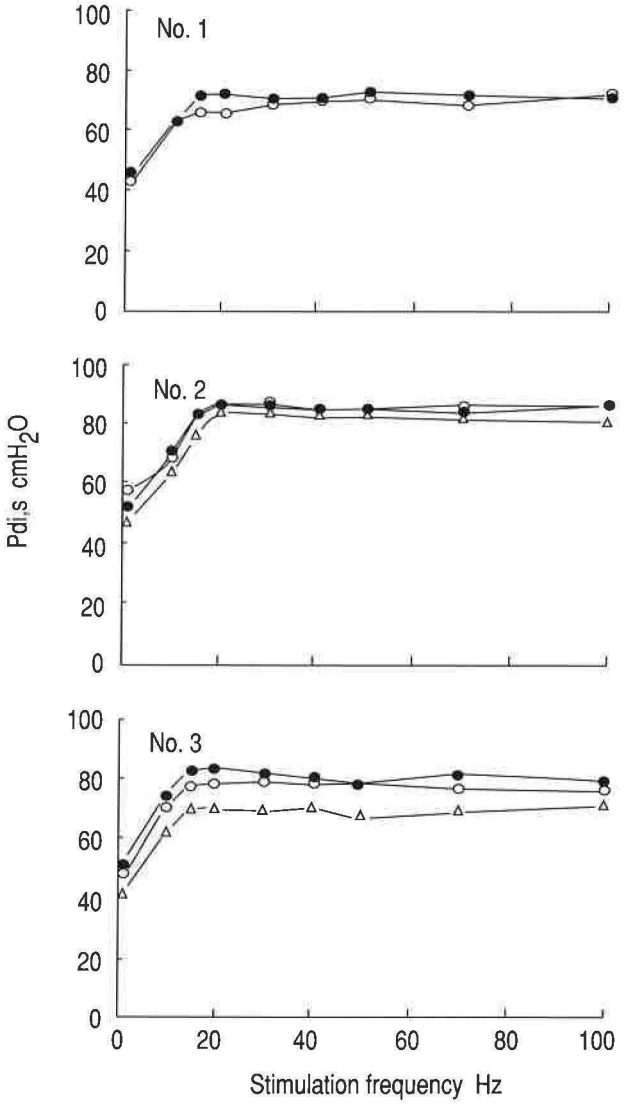

Fig. 3. – Transdiaphragmatic pressure swing (Pdi,s)-frequency curves in response to bilateral phrenic nerve paired stimuli from three subjects (different symbols), when the test was repeated on separate days over 1–20 weeks.

No significant difference was found on repeated trials (ANOVA $p<0.8$); the small between-day variability presumably being accounted for by slight differences in the degree of abdominal binding.

As shown in figure 4A, Pdi,s increased between 10 and 20 Hz. Further increase in stimulation frequency did not result in greater Pdi,s. Pdi,s decreased at all frequencies after fatigue (ANOVA $p<0.0001$) and recovered only minimally after 15 min rest. The ratio of Pdi,s at 10 Hz over that at 100 Hz ($Pdi,s_{10/100}$) decreased from 0.88±0.03 before fatigue to 0.76±0.05 immediately after the fatigue run ($p<0.05$) and to 0.68±0.05 after 15 min rest ($p<0.01$).

Figure 4B shows that in the fresh state, the amplitude of T2 decreased with increasing stimulation frequency. The shape of this relationship was markedly affected by fatigue: fatigue preferentially depressed T2 amplitude at low stimulation frequencies, which recovered slowly. By contrast, the small decay of T2 at high frequencies recovered quickly.

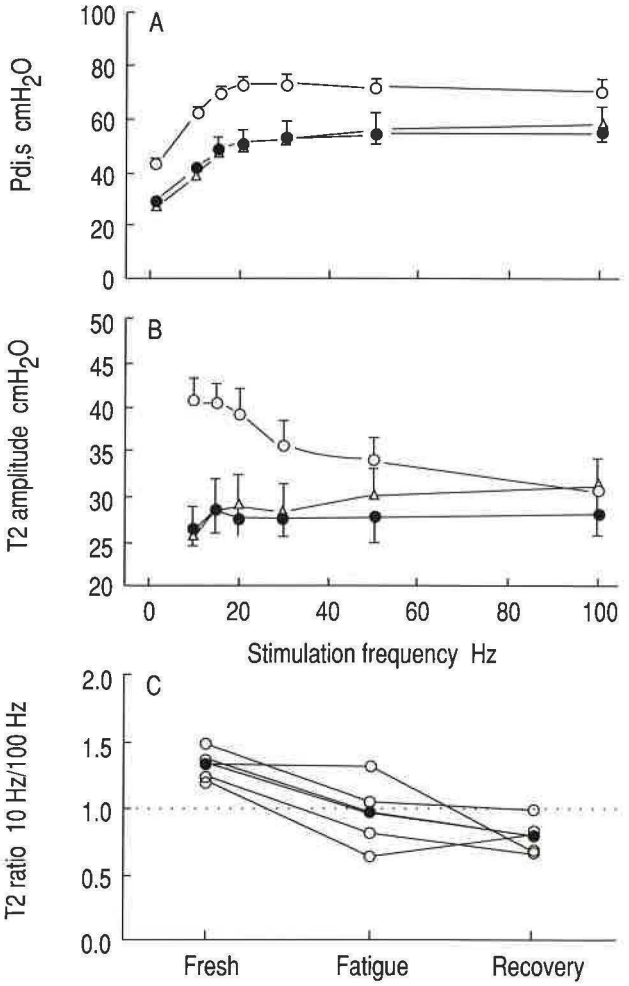

Fig. 4. – A) Group mean transdiaphragmatic pressure swing (Pdi,s)-frequency curves in response to bilateral phrenic nerve paired stimuli before (○), after fatigue (●), and after 15 min recovery (Δ). B) Group mean amplitude of the second twitch (T2) plotted as a function of stimulation frequency before (○), after fatigue (●), and after 15 min recovery (Δ). C) Ratio of T2 10 Hz over that at 100 Hz ($T2_{10/100}$) before, after fatigue, and after 15 min recovery. Open circles are for individual data and closed circles for group mean. Dotted line is line of equality.

Because of the preferential fall of T2 at low frequencies and its quick recovery at high frequencies, the amplitude ratio of T2 at 10 Hz over 100 Hz ($T21_{10/100}$) decreased significantly from a control value of 1.33±0.05 to 0.97±0.12 ($p<0.025$) immediately after the fatigue run, and to 0.81±0.06 ($p<0.001$) after 15 min rest (fig. 4C).

Animal study

The contractile properties obtained from rat diaphragm strips in fresh condition (table 1) were comparable to previously published values at 25°C [20, 21]. The force-frequency curves of fresh diaphragm strips from paired shocks, from four pulses, and from trains of pulses are shown in figure 5. In all cases, the force increased with increasing stimulation frequency. At any frequency, the force also increased with increasing number of stimuli, especially at high frequencies. The frequency at which the force became maximal also increased with increasing number of stimuli. Consistent with the human *in vivo* data of this study, the amplitude of T2 calculated in the same manner from paired responses progressively decreased with increasing frequency of stimulation.

The effects of fatigue and recovery on the force of single twitch, paired shocks, and tetanic stimulation at 10 and 100 Hz are summarized in table 2.

Table 1. – Contractile properties of rat diaphragm strips

n	Pt $N\cdot cm^{-2}$	CT ms	1/2RT ms	Po $N\cdot cm^{-2}$	Pt/Po
9	10.49 ±0.41	39.4 ±1.0	47.0 ±1.8	23.83 ±1.01	0.44 ±0.01

Values are mean±SEM (n=9). Pt: single twitch tension; CT: contraction time; 1/2RT: half relaxation time; Po: maximal isometric tension (recorded at 100 Hz and at optimal sarcomere length).

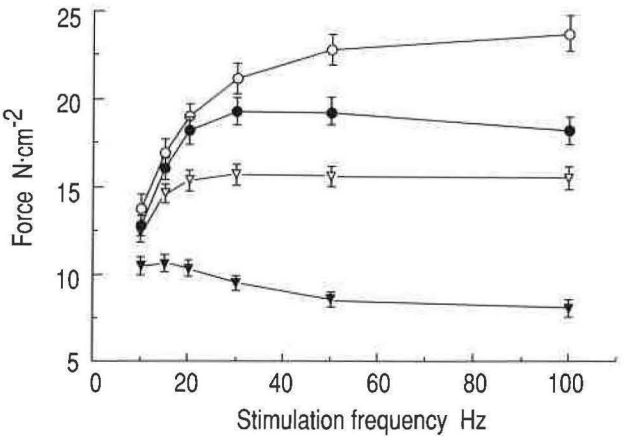

Fig. 5. – Force-frequency relationships of fresh rat diaphragm strips with tetanic stimulation (○), four shocks (●), paired shocks (▽), and T2 response to paired shocks (▼).

All responses decreased with fatigue, though more at low than at high stimulation frequencies, and all showed the tendency to recover during the 30 min recovery period. As shown in figure 6, $T2_{10/100}$ and tetanic force ratio at 10 Hz over 100 Hz ($P_{10/100}$) changed in a similar way with fatigue and recovery ($p<0.005$). When $T2_{10/100}$ and $P_{10/100}$ are plotted one against another under all conditions, a highly significant correlation ($r=0.94$, $p<0.001$) was found (fig. 7). Fatigue and subsequent recoveries shifted this relationship towards lower values.

Table 2. – Effect of fatigue and recovery on force output in rat diaphragmatic strips, in response to different stimuli

	Fresh	Fatigue	Recovery 15 min	Recovery 30 min
Pt	10.05±0.29	4.87±0.17#	5.85±0.21#	6.51±0.22§
Pa_{10}	11.73±0.35	5.95±0.28#	6.94±0.37§	7.76±0.41§
Pa_{100}	15.17±0.47	8.43±0.29#	10.30±0.28§	11.59±0.24†
P_{10}	12.55±0.26	6.78±0.33#	7.33±0.44#	8.16±0.50#
P_{100}	23.38±0.88	14.89±0.41§	18.48±0.43†	20.60±0.57*

Values are mean±SEM (n=6) and expressed as $N\cdot cm^{-2}$. Pt: single twitch; Pa_{10}: paired twitches at 10 Hz; Pa_{100}: paired twitches at 100 Hz; P_{10}: tetanic force at 10 Hz; P_{100}: tetanic force at 100 Hz. Compared with fresh state, *: $p<0.05$; †: $p<0.01$; §: $p<0.001$; #: $p<0.0001$.

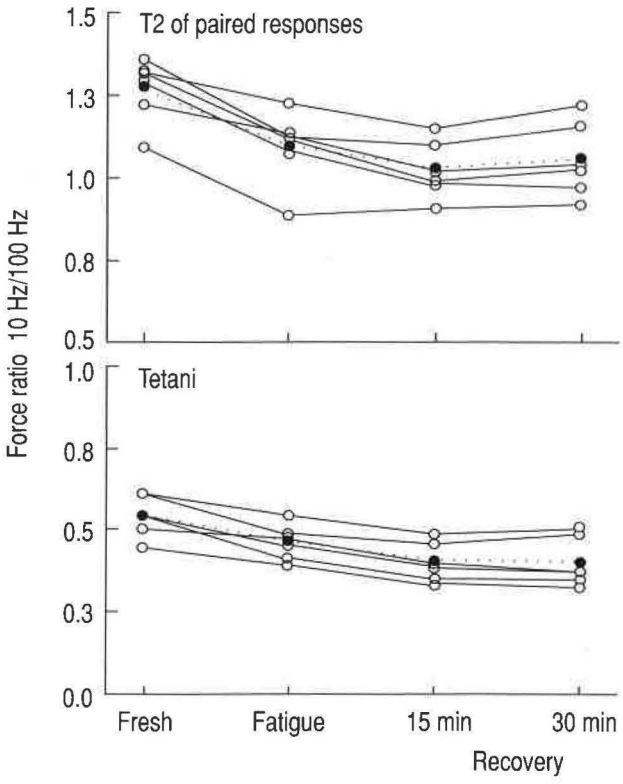

Fig. 6. – For the rat diaphragm, ratio of T2 (upper panel) and of tetanus (lower panel) at 10 Hz over that at 100 Hz before, after fatigue, and following recovery. Open circles, solid line are for individual data and closed circles, dotted line for group mean.

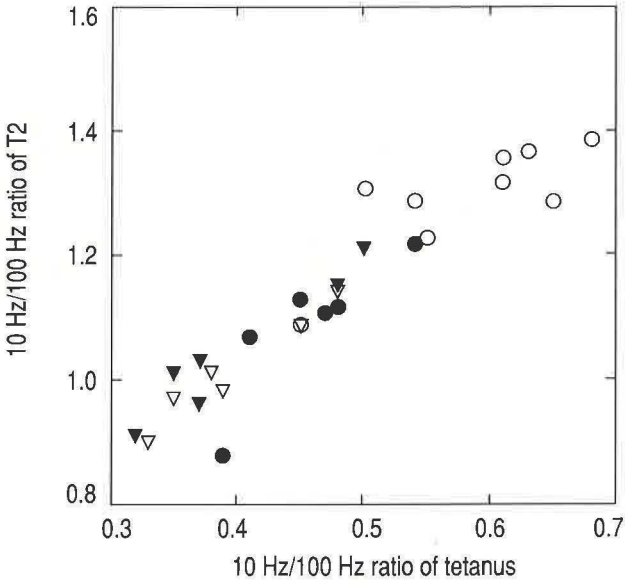

Fig. 7. – For the rat diaphragm, ratio of T2 plotted as a function of ratio of tetanus at 10 Hz over 100 Hz before (○), after fatigue (●), after 15 min recovery (▽), and after 30 Min recovery (▼).

Discussion

Force-frequency relationship of the diaphragm

As shown for the *in vitro* rat diaphragm preparations, the force-frequency relationship differs depending on the number of impulses in the train (fig. 5). Comparable differences can be found for the human *in vivo* diaphragm, by comparing the Pdi-frequency relationships obtained here using paired stimuli with those previously reported using trains of tetanic stimulation [4, 9, 11]. Compared with single twitches, paired stimuli elicited additional Pdi or force which was expressed by T2. With both *in vitro* rat and *in vivo* human diaphragm preparations the T2 amplitude decreased progressively with increasing frequency, as could be expected based on the characteristic shape of the force-frequency relationships for both muscles [4, 9, 22]. Without this decrease, the Pdi or force during a long tetanus would not tend towards a finite maximal value as frequency increases.

Effect of fatigue and recovery

Peripheral fatigue of skeletal muscles has been conventionally subdivided into low and high frequency fatigue components, based on the different rate at which the force recovers when measured in response to low (10–20 Hz) or high (50–100 Hz) stimulation frequencies [1–3]. Both types of fatigue have been documented previously in normal human diaphragm *in vivo*, using unilateral phrenic nerve tetanic stimulation [4, 9]. Low frequency fatigue of the respiratory muscles has been thought to have important clinical significance for the following reasons. Firstly, there is always a preferential loss of force in the low frequency range when skeletal muscles do intense exercise [4, 6, 9]. Secondly, low frequency fatigue generally lasts for many hours after the cessation of the fatiguing task [2, 4, 6]. Thirdly, the firing frequency of skeletal muscles for daily activities is within the low frequency range [1, 23]. Finally, increasing severity of low frequency fatigue is associated with progressively decreased endurance time [7].

Low frequency fatigue of the diaphragm, like that of other skeletal muscles, can be identified by calculating the ratio of Pdi generated at a low frequency (generally 20 Hz) over that generated at a high frequency (50 or 100 Hz) [4, 6, 9]. This ratio is normally quite stable, but decreases significantly following a diaphragmatic fatiguing task [4, 8, 9]. The present study, using bilateral phrenic nerve paired shocks, demonstrated that, as expected, fatigue decreased Pdi,s at all stimulation frequencies. There was also a less pronounced but statistically significant decrease in $Pdi,s_{10/100}$ with fatigue. However, in contrast to previous studies, the Pdi response to high frequency stimuli only recovered a little after 15 min rest (fig. 4A).

These differences are best explained when considering the Pdi response to paired stimuli as the sum of two successive responses (T1+T2), the first being equal to a single twitch and independent of the stimulation frequency. Low frequency fatigue leads to a reduction in twitch amplitude [24, 25]. The reduction in the size of the single twitch and hence of T1 in our subjects, due to the presence of low frequency fatigue, would have a carry-over effect at all stimulation frequencies, thus explaining both the relatively smaller decrease of $Pdi,s_{10/100}$ with fatigue and the persistent decline of the paired responses even at high frequencies after 15 min rest. This interpretation is supported by the progressively increasing recovery of T2 with increasing stimulation frequency after 15 min rest in the human study (fig. 4B). Because of this characteristic of paired twitches, $Pdi,s_{10/100}$ would be less sensitive to reflect low frequency fatigue compared with tetanic stimulation, from which the carry-over effects of the first twitch would be very small. However, this in turn raises the possibility that the clearly distinct rates of recovery of T2 amplitude at low and high frequencies carries the same information as the ratio of Pdi at 20 Hz to that at 50 or 100 Hz, obtained from tetanic phrenic stimulation. Both may be of equal value in demonstrating low frequency diaphragmatic fatigue in humans. Our results from *in vitro* rat diaphragm strips show that this is indeed the case (figs 6 and 7). Furthermore, the bilateral T2 measurements at 10 and 100 Hz are tolerable, whereas bilateral supramaximal tetani at these frequencies are not [11].

As shown in figure 4C, there was a considerable decrease in $T2_{10/100}$ ratio with fatigue and after 15 min recovery. The only exception was from one subject, whose $T2_{10/100}$ did not change immediately after fatigue run, which could be explained, at least in part, by

twitch potentiation following an intense muscular work in performing the fatiguing task [26]. For this subject, $T2_{10/100}$ ratio decreased to 50% of the post-fatigue value after 15 min rest. This strongly suggests that the decrease of $T2_{10/100}$ ratio can be masked by twitch potentiation immediately following the cessation of a fatiguing task in some individuals. Simply repeating the measurements after 15 min, not only makes this test more sensitive, because twitch potentiation is no longer a confounding variable, but (in the limited number of subjects we studied) also provides a value in which there is no overlap between fresh and fatigued conditions. Our data suggest that a single value of $T2_{10/100}$ smaller than 1.1 indicates the presence of low frequency fatigue. Whether a single value of $T2_{10/100}$ can be used to diagnose low frequency fatigue needs further investigation. In this connection, a number of indices including maximal inspiratory pressure [27], single Pdi twitch amplitude [28], power spectrum analysis [29], and relaxation characteristics of voluntary and stimulated Pdi [30] have been used to evaluate human diaphragm fatigue and have been suggested as clinically useful diagnostic tests. Unfortunately, interpretation of all of these indices requires at least two measurements over time, because there is considerable overlap of the values from the fresh to the fatigued state, so that a comparison of the fatigued with the fresh or recovered value is necessary in order to have diagnostic significance, which makes these measurements inconvenient and time-consuming. An index of low frequency fatigue which does not need repeated determinations is, therefore, highly desirable for clinical purpose. The $T2_{10/100}$ ratio may meet this criterion.

That we found a considerable overlap of $T2_{10/100}$ ratio between fresh and fatigued *in vitro* rat diaphragm strips is not surprising, since many differences are believed to exist between human *in vivo* and rat *in vitro* studies. As has been suggested by many authors [31–33], the most important differences may be the fatigue protocols of *in vitro* and *in vivo* studies and their different rate of recovery, particularly at high frequencies. Thus, the fact that there is overlap between the fresh and fatigued values in rats does not influence our hope that a single measurement of $T2_{10/100}$ in humans may be diagnostically useful.

Technical considerations

Paired stimuli have previously been used in limb muscles to study the correlation between twitch contraction and force summation [34], the active state [35, 36], and the isometric contraction of a single motor unit [37]. To our knowledge, however, this has not been used previously in the study of respiratory muscles. In terms of reproducibility, it has been shown that diaphragmatic single twitches in response to supramaximal phrenic nerve stimulation are highly reproducible for a given subject, when the determinations are made on separate days [14, 38]. We have shown, in the present study, that this is also the case for diaphragmatic paired twitches (fig. 3).

A requirement of proper paired twitch measurement is that the abdomen and lower rib cage need to be restricted to reduce the shortening of the diaphragm [14, 39] as much as possible. For clinical applications, this procedure needs to be further standardized.

In summary, phrenic nerve paired shocks provide similar information to tetanic phrenic stimulation, in terms of diaphragmatic contractility and function. The technique can be performed bilaterally with reproducibility and is well-tolerated by normal subjects, so that it is more applicable than tetanic phrenic nerve stimulation for the study of diaphragmatic contractility. Our results showed that the $T2_{10/100}$ ratio is a valuable index for assessing diaphragmatic low frequency fatigue, a finding well supported by *in vitro* rat diaphragmatic strip studies. However, further evaluation of the technique is needed before it can be recommended for clinical use, due to the relatively small number of subjects tested in the present study.

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