The Journal of Experimental Biology 207, 4505-4513
Published by The Company of Biologists 2004
doi:10.1242/jeb.01259

Enhancement of twitch force by stretch in a nerve–skeletal muscle preparation of the frog *Rana porosa brevipoda* and the effects of temperature on it

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Accepted 23 August 2004

Summary

We investigated the mechanism of the enhancement of twitch force by stretch and the effects of temperature on it in nerve–skeletal muscle preparations of whole iliofibularis muscles isolated from the frog *Rana brevipoda*. When a preparation was stimulated indirectly and stretched, the twitch force after the stretch was enhanced remarkably in comparison to that observed before a stretch at low temperature. The enhanced force obtained by a stretch of 20% resting muscle length (l_0) at low temperature was as high as the force obtained by direct stimulation. The phenomenon was not dependent on the velocity but on the amplitude of stretch. The enhanced force obeyed the length–force relationship when a stretch was long enough. The above results were observed when the frogs were kept at room temperature (20–22°C). Measurements were also taken at low temperature (4°C); when frogs were kept at low temperature for more than 2 months, twitch force obtained without stretch was considerably higher at l_0. The amplitude of the action potential recorded extracellularly from the muscle surface increased remarkably after a stretch, but was same before and after a stretch when recorded from the nerve innervating muscle. The effects of temperature on twitch and tetanic force by direct or indirect stimulation without stretch were also studied as basic data of the stretch experiment. The results from this study suggest that stretch-induced force enhancement in a nerve–muscle preparation is caused by an increase in the transmission rate between nerve and muscle, and the amplitude of the enhanced force is determined by the length–force relationship of the muscle. The phenomenon is also strongly affected by the temperature at which the frogs are kept.

Key words: stretch, force enhancement, nerve–muscle preparation, synaptic transmission, thermal acclimation, nerve stimulation, frog, *Rana brevipoda*.

Introduction

Many investigators have studied the mechanical properties of skeletal muscle with regard to the relationship between contractile force and stretch in length during muscle activity. Abbott and Aubert (1952) first showed on toad sartorius muscles and dogfish jaw muscles without a nerve that a stretch during tetanus causes a remarkable increase in force. The stretch-induced force enhancement during tetanic activity comprises three different phases: (1) an initial rapid rise of force, (2) a slow rise following the first one during stretch ramp, and (3) maintenance of the force at a higher level than that recorded in the corresponding isometric contraction at the stretched new length (Edman et al., 1978). Edman et al. (1978) suggested that during stretch ramp, the amplitude of the increase in force was dependent on the velocity of stretch, and the residual force enhancement after a stretch was critically dependent on the stretch amplitude. This phenomenon has been further explored in subsequent studies (Edman et al., 1982; Edman and Tsuchiya, 1996; Herzog and Leonard, 2002; Herzog et al., 2003; Mansson, 1989; Sugi and Tsuchiya, 1988), which suggested that the stretch-induced force correlated with cross-bridge performances or passive elements in sarcomeres.

The propagation mechanism of excitation in the nerve–muscle junction is known to be chemical transmission, where transmitter substances at synapses are released from the synaptic vesicles accumulated within the nerve endings (Katz, 1996), and the effects of various factors on nerve propagation have been reported; for example, the dependence of transmission efficiency on temperature (Adams, 1989; Balnave and Gage, 1974; Barrett et al., 1978; Barrett and Stevens, 1972; Barton and Cohen, 1982; Katz and Miledi, 1965; Molgo and Van der Kloot, 1991; Van der Kloot and Cohen, 1984) and on muscle length (Chen and Grinnell, 1995, 1997; Fatt and Katz, 1952; Hutter and Trautwein, 1956; Ruff, 1996, 2003; Turkanis, 1973; Ypey et al., 1974; Ypey and Anderson, 1977). Most experiments, however, have been performed under conditions where muscle movement was inhibited by a blocker such as curare or by specific ionic conditions, and the mechanical

phenomenon has only been mentioned briefly in a few papers (Chen and Grinnell, 1997; Hutter and Trautwein, 1956).

In the present study, we intended to explore new aspects of force enhancement by stretch using nerve–skeletal muscle preparations isolated from frogs, firstly because the phenomenon seemed to be closely related to physiological functions *in situ* and, further, because we discovered in early stages of the experiment that the phenomenon was remarkably affected by the temperature at which the frogs were kept, i.e. the thermal acclimation.

In the present study we show that the stretch-induced force enhancement in a nerve–muscle preparation is caused by the increased transmission rate between nerve and muscle and that this phenomenon is strongly affected by the temperature at which the frogs were kept.

Materials and methods

Muscle preparation and mounting

Frogs *Rana porosa brevipoda* Ito were obtained from a supplier who collected the animals from the suburbs of Tokyo in Japan. They were separated into two groups; one group was kept at room temperature (22°C) and the other at 4°C. All the procedures were carried out in accordance with Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. Nerve–skeletal muscle preparations were made from iliofibularis muscle and the sciatic nerve. Some experiments were performed on muscle preparations without nerve as controls. Metal hooks were fixed tightly by cotton thread at either end of a preparation, which was mounted horizontally in the experimental bath made of brass with an inner surface coated by silicone rubber. The hooks were connected to the arms of a force transducer and a servomotor and the sarcomere length was carefully adjusted to 2.2 μm at rest by measuring the diffraction of a He–Ne laser (05-LHR-121, Melles Griot, Carlsbad, CA, USA) on a screen 15 cm away from the muscle (Edman, 1975) (Fig. 1A). The preparation was bathed in Ringer solution containing (in mmol l^{-1}): NaCl, 115.5; KCl, 2.0; $CaCl_2$, 1.8; Na_2HPO_4+NaH_2PO_4, 2.0; pH 7.0 (Edman, 1975). The solution was circulated through the bath at a rate of 3 ml min^{-1} using a peristaltic pump with thermo-electric device for temperature control (CTR-120, Komatsu-Yamato, Tokyo, Japan). The experiments were carried out at three different temperatures (4±0.5, 12±0.5 and 22±0.5°C).

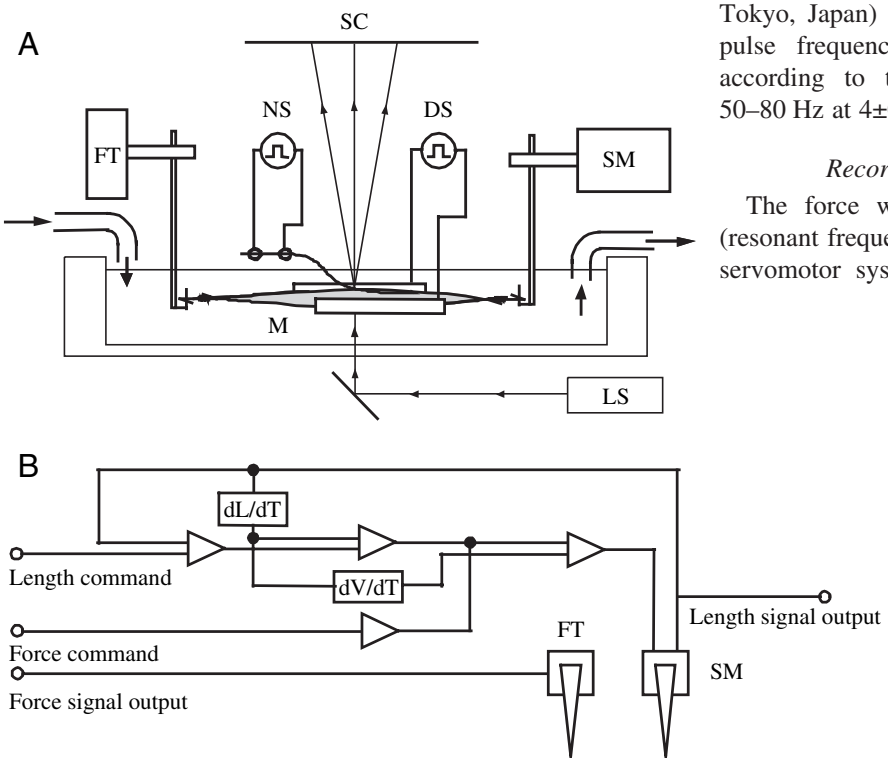

Fig. 1. Schematic illustrations of experimental setup. (A) An experimental bath containing a muscle preparation. DS, stimulator for direct stimulation; FT, force transducer; LS, He–Ne laser for monitoring sarcomere length; M, whole muscle preparation; NS, stimulator for nerve (indirect) stimulation; SC, screen for monitoring laser diffraction pattern; SM, servo-motor. The circulation of physiological saline is shown by thick arrows. The path of the laser light is shown by thin arrows. (B) Diagram of the electronic circuit used to drive a dual-mode servomotor (SM) for controlling muscle length and load and for recording muscle force. Force change was also measured by a force transducer (FT).

Stimulation

Electronic stimulation to contract the muscle was applied either on the nerve (indirect stimulation) or on the muscle (direct stimulation). Indirect stimulation was carried out using a pair of platinum circular wires (separated by 3 mm) around a nerve bundle. The surface of the nerve between the two electrode wires was filled with the mixture of vaseline and oil for effective stimulation. For direct stimulation a pair of platinum plate electrodes was placed either side of a muscle.

A single pulse or a train of pulses generated by an electronic stimulator (SEN-3301, SEN-7203, Nihon Kohden, Tokyo, Japan) was delivered to induce a twitch or a fused tetanus of 1 s duration. For direct stimulation, the Ringer solution contained curare (*d*-tubocurarine chloride pentahydrate, 10^{-4} mol l^{-1}). Rectangular pulses (0.03–1.0 ms in duration) of sufficient voltage, twice as strong as the threshold level, were applied through an isolator (SS-104J, SS-202J, Nihon Kohden, Tokyo, Japan) for both direct and indirect stimulation. The pulse frequency for tetanus was changed appropriately according to the temperature (20–25 Hz, 35–45 Hz and 50–80 Hz at 4±0.5, 12±0.5 and 22±0.5°C, respectively).

Recording of signals and stretch of muscle

The force was measured either by a force transducer (resonant frequency, 1 kHz) or by the feedback signal from a servomotor system (DPS-265, Diamedical System, Tokyo,

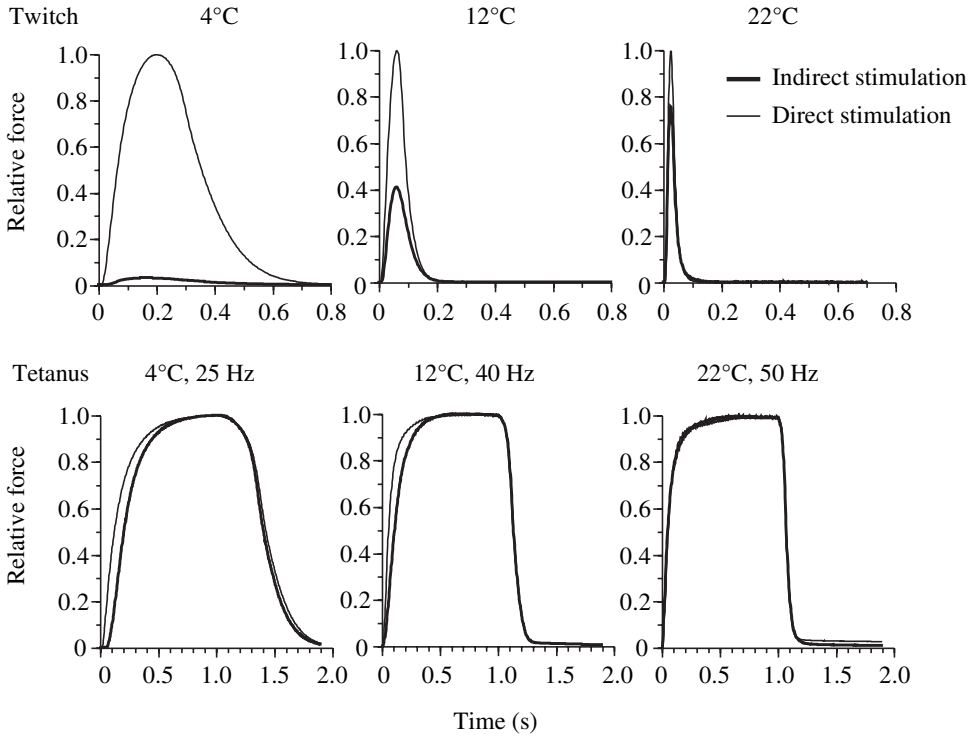

Fig. 2. Typical recordings of twitch and tetanic forces developed by direct or indirect stimulation at three different temperatures, 4°C, 12°C and 22°C. Twitch and tetanic forces at the different temperatures were recorded from the same preparations. The duration of the stimulation in tetanus was 1 s. Forces obtained by indirect stimulation (thick lines) are expressed relative values to those obtained by direct stimulation (thin lines) at each temperature. Temperatures and stimulation frequencies in tetanus are denoted for each recording.

Japan) (Fig. 1B). The servomotor was also used to apply step increases in length from the resting length (l_0) and a stretch was applied in between twitches. The time for a length step was usually 5 ms, but was changed appropriately when a slow-ramp stretch was necessary. On application of a stretch, the twitch force amplitudes were measured after the passive resting force had become constant. The force per cross-sectional area was estimated by calculating the area from the long and short diameters of a whole muscle, assuming that the cross-section of iliofibularis muscle was elliptical in shape. Forces developed by indirect stimulation were expressed as values relative to the forces obtained by direct stimulation.

The signals from force and displacement transducers were displayed simultaneously on digital oscilloscopes (Model 310, Nicolet, Madison, WI, USA; DL716, Yokogawa, Tokyo, Japan) and a pen recorder (RTA-1100M, Nihon Kohden, Tokyo, Japan). To record the extracellular electrical signals from sciatic nerve or muscle, a suction electrode made of polyethylene tube (Suction electrode, A–M Systems, Carlsborg, WA, USA) was used, sucked onto the region near the tendon on the muscle surface for less movement, or on the surface of the nerve bundle, and another electrode was placed in the bath. The electrical signals were amplified by an extracellular amplifier (DPA-1000, Diamedical, Tokyo, Japan) and displayed on a digital oscilloscope.

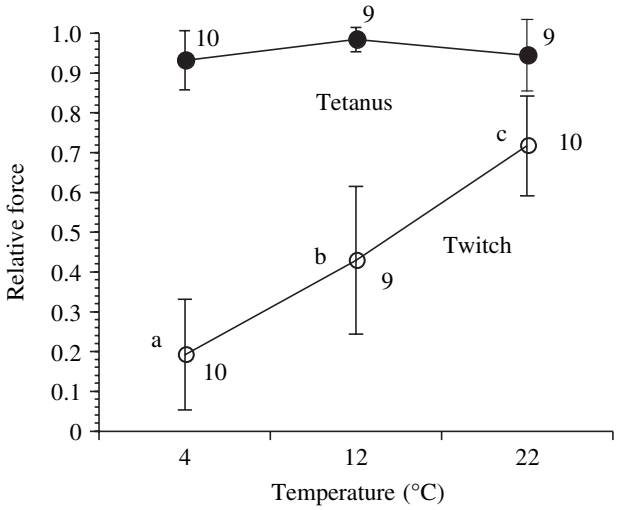

Fig. 3. Change in twitch and tetanic force developed by indirect stimulation with a change in temperature. Twitch (open circles) and tetanic (closed circles) forces are expressed relative to the respective forces obtained by direct stimulation. Values are means ± S.D. The figures at each data point denote the number of samples used. The small letters (a, b and c) in twitch denote that the differences between points are statistically significant (P<0.01), while the differences in tetanus are not significant.

Statistics

Values in the text and the figures are given as the mean ± standard deviation (S.D.). Statistical differences between the values were determined by either Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Scheffé *post hoc* test.

Results

Effects of temperature on twitch and tetanic force

We first examined the effects of temperature on twitch and tetanic force induced by direct and indirect stimulation at three different temperatures. Tetanic forces were produced by the different stimulation frequencies at different temperatures to obtain complete fusion of force. The twitch force per cross-

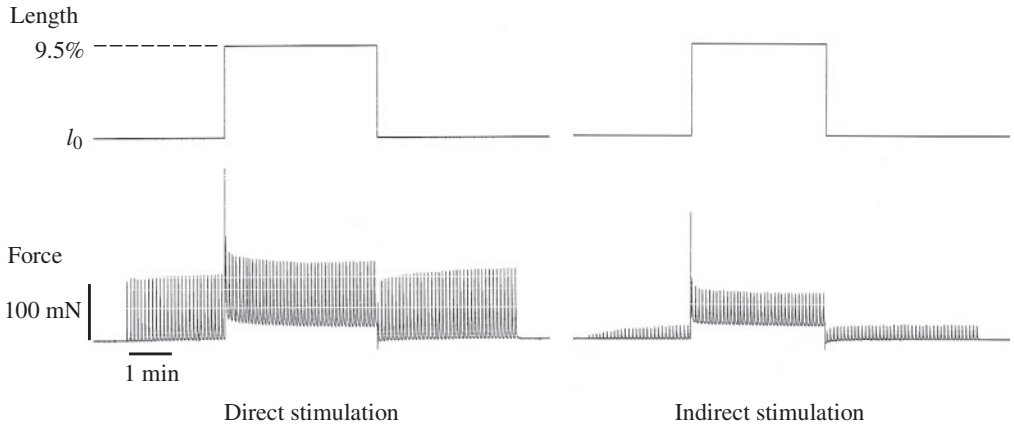

Fig. 4. Changes in twitch force induced by stretch in directly and indirectly stimulated muscle. (Top) Length steps applied; (bottom) force changes. The muscle was stimulated at 5 s intervals at 4°C and stretched by 9.5% l_0 (muscle length) from l_0 in the course of train twitch stimuli. All recordings were obtained from the same preparation.

sectional area obtained by indirect stimulation was significantly different at different temperatures: 9.84±8.36 mN mm^{-2} (N=5) at 4°C and 62.04±35.64 mN mm^{-2} (N=5) at 22°C (P<0.05). The tetanic force per cross-sectional area obtained by indirect stimulation was 161.01±24.56 mN mm^{-2} (N=5) at 4°C and 210.81±50.73 mN mm^{-2} (N=5) at 22°C and there was no significant difference between them.

We compared the amplitude of force produced by indirect stimulation with that obtained by direct stimulation (Figs 2 and 3). The twitch force obtained by indirect stimulation was approximately one tenth of that from direct stimulation at 4°C, while it was 40% and 70% at 12°C and 22°C, respectively (Fig. 3). The differences between them were significant (P<0.01). By contrast, tetanic forces obtained by direct and indirect stimulation at each temperature were not different, though the rate of force development was slower on indirect stimulation at the lower temperature (Fig. 2). Thus the intensities of direct and indirect stimulations were sufficiently strong to activate all nerve and muscle fibres.

Enhancement of twitch force by stretch

Fig. 4 illustrates the changes in twitch force on application of a stretch. The stretch was applied in between twitches, that is, to silent muscle, as shown in Fig. 7A. On indirect stimulation, higher twitch forces than those obtained before a stretch superimposed on the resting force were observed after a stretch, and they returned immediately to base level after the end of a stretch. On direct stimulation, however, the forces did not change appreciably before and after a stretch.

We examined the relationship between muscle length and force production in directly and indirectly stimulated muscles (Figs 5 and 6). On direct stimulation, the twitch forces decreased at all temperatures at longer lengths than at the resting length (l_0) (Fig. 5A) and were similar to those seen in tetanus (Fig. 5B). By contrast, on indirect stimulation at 4°C (Fig. 6), the enhanced forces increased with the increase in a length step and approached the regression line of the length–force relationship at approx. 1.2× l_0. At 12°C, the enhanced forces were higher at 1× l_0 and approached the regression line at around 1.10–1.15× l_0. At 22°C, very high relative forces (0.6–0.8) were developed even at l_0 and approached the regression line at 1.05× l_0, after which the force decreased along the regression curve at longer lengths. Thus enhanced forces increased with the stretch amplitudes but

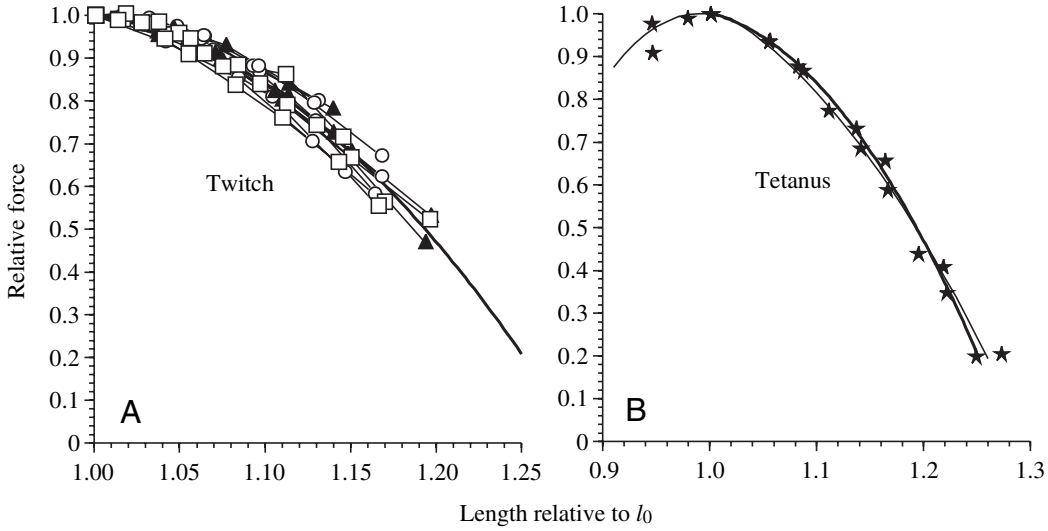

Fig. 5. Length–force relationships in twitch (A) and tetanus (B) in directly stimulated muscle. Forces are expressed as values relative to the force at one muscle length (l_0). In A, each symbol shows the data obtained at three different temperatures; open circles, 4°C (N=5); filled triangles, 12°C (N=4); open squares, 22°C (N=4). The thick line shows the least-squares regression curve calculated from the total data in twitch. In B, the data were obtained from three different preparations all at 4°C. The thin line is the regression curve obtained from three preparations. The thick line is the same regression curve as in A.

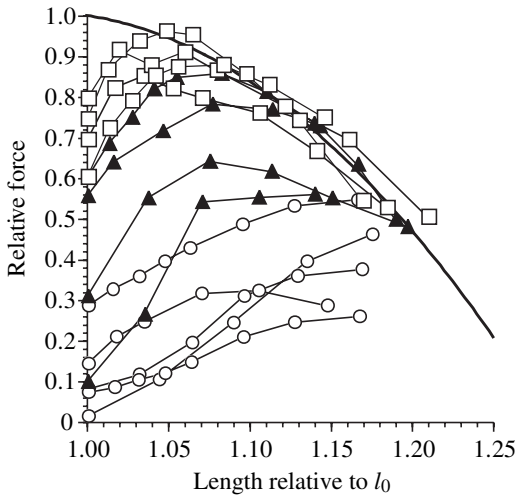

Fig. 6. The effects of temperature on stretch-induced enhancement of twitch force in indirectly stimulated muscle. Twitch forces are expressed as values relative to the force obtained by direct stimulation at one muscle length (l_0). The thick line is the same regression curve as shown in Fig. 5A. Forces were measured 2 min after stretching each preparation from l_0 to the length indicated on the abscissa at three different temperatures; open circles, 4°C (N=5); filled triangles, 12°C (N=4); open squares, 22°C (N=4). All animals had been kept for more than 2 months at room temperature before the experiment.

followed the length–force relationship and decreased at long lengths.

Effects of velocity of stretch on twitch force

It is known that the residual force after stretch in tetanus depends not on the velocity but on the stretch amplitude (Edman et al., 1978, 1982). In the present experiment, the effects of stretch velocity on twitch force enhancement were examined (Fig. 7). Two stretches at different velocities (fast and slow) from the resting length (l_0) were applied to a muscle between twitches (Fig. 7A). The amplitude of enhanced twitch forces following the two different stretches is almost same (Fig. 7B). Fig. 7C shows that the form of the force development is the same for both stretches, which suggests that the enhancement of twitch force is independent of stretch velocity.

Effects of the maintenance temperature of frogs on force enhancement

The results discussed so far were obtained from frogs kept at room temperature. We attempted to keep frogs at a low temperature (4°C) for more than 2 months and the experiments were conducted at this low temperature. The responses shown in Fig. 8 obtained from the frogs kept at 4°C were very similar to those shown in Fig. 6 at the experimental temperature of 22°C from frogs kept at room temperature.

Twitch forces at the resting length were compared after different periods of maintenance at 4°C (Fig. 9). It is clearly demonstrated that the twitch forces were higher when frogs had been kept for longer periods at 4°C. The force obtained from frogs kept at 4°C for more than 2 months was significantly higher than that from frogs kept at room temperature (P<0.01).

Electrical signals from nerve and muscle

The results so far described seem to be explained either by a change in conduction at the neuromuscular junction or by a change in the excitability of nerve itself. We therefore measured the electrical signals from nerve and muscle (Fig. 10). In muscle (Fig. 10B), significantly higher action potentials (15.00±3.29 mV) were observed after a stretch than before (4.83±0.71 mV) (P<0.01), whereas the heights of the action potentials observed in nerves before and after a stretch at 4°C were the same (Fig. 10A).

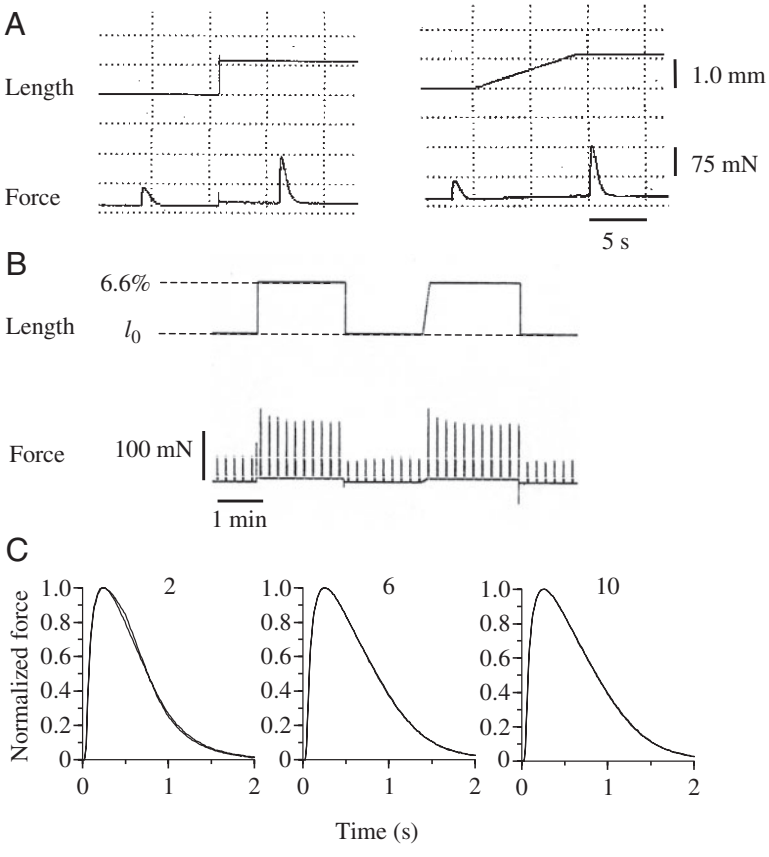

Fig. 7. The influence of stretch velocity on the enhancement of twitch force induced by indirect stimulation. (A) A quick (4 ms) or slow (8 s) lengthening step of 6.6% l_0 was applied to a muscle (top trace) and the twitch forces were compared before and after the stretch (lower trace). (B) The slow-time recordings of the same data as in A. Twitch stimuli were applied every 12 s. (C) Comparison of the enhanced forces obtained by fast and slow stretch. The recordings obtained by fast and slow stretches are shown together in each panel (the second, sixth and tenth records after stretch) but the two recordings are almost superimposed.

Discussion

Basic mechanism of the enhancement of twitch force in nerve–muscle preparation

Force enhancement by stretch during tetanus has previously been studied using preparations of a single muscle fibre without nerve or a whole muscle with nerve (Abbott and Aubert, 1952; Edman et al., 1978, 1982; Edman and Tsuchiya, 1996; Herzog and Leonard, 2002; Herzog et al., 2003; Mansson, 1989; Sugi and Tsuchiya, 1988). However, there are only a few studies on the enhancement of twitch force in nerve–skeletal muscle preparations (Chen and Grinnell, 1997; Hutter and Trautwein, 1956). Some electrophysiological experiments performed in nerve–muscle preparations demonstrated that the increases in amplitude of end-plate potential (EPP) and the frequency of miniature end-plate potential (mEPP) were brought about by lengthening muscle (Chen and Grinnell, 1995, 1997; Hutter and Trautwein, 1956; Turkanis, 1973; Ypey and Anderson, 1977), but these experiments were carried out under conditions where the conduction was partially blocked at the neuromuscular junction. In the present study we have elucidated the mechanism of force enhancement by stretch from a new viewpoint.

A quick stretch enhanced twitch force when muscle was stimulated indirectly, whereas the enhancement was not observed in directly stimulated muscle (Fig. 4). Therefore, stretch was thought to cause the force enhancement by changing the conduction between nerve and muscle. The preparations used in the present experiment were whole

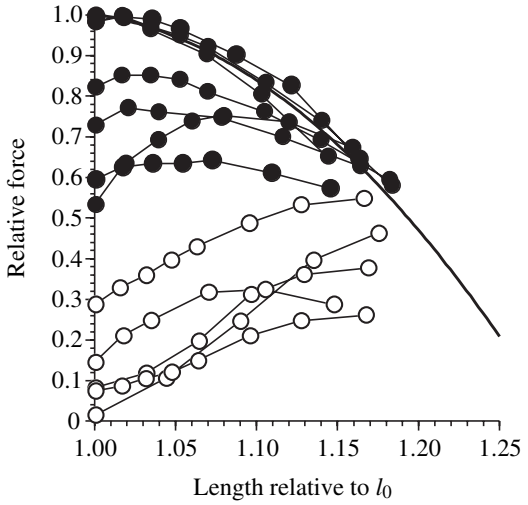

Fig. 8. The influence of maintenance temperature of frogs on the enhancement of twitch force by stretch. Frogs were kept at room temperature (22°C, open circles) or at 4°C (filled circles) for more than 2 months and twitch forces were measured at low temperature (4°C) in both cases. Forces were measured 2 min after stretching a muscle from l_0 to the length indicated on the abscissa. The data points (open circles) are the same as those shown in Fig. 6. Twitch forces are expressed as values relative to the force at resting length (l_0) by direct stimulation. The thick line shows the regression curve of the twitch force induced by direct stimulation as shown in Fig. 5A.

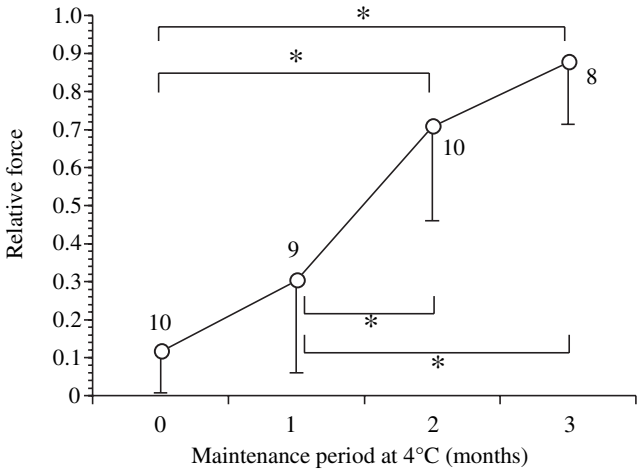

Fig. 9. The thermal acclimation of twitch force by indirect stimulation. Frogs were kept for 0, 1, 2 or 3 months at 4°C and twitch forces were measured at resting muscle length (l_0) at 4°C. Twitch forces are expressed as values relative to those obtained by direct stimulation at l_0 at 4°C. The numbers at data points indicate the number of preparations used. *Differences between two data points marked by horizontal lines are statistically significant by $P<0.01$.

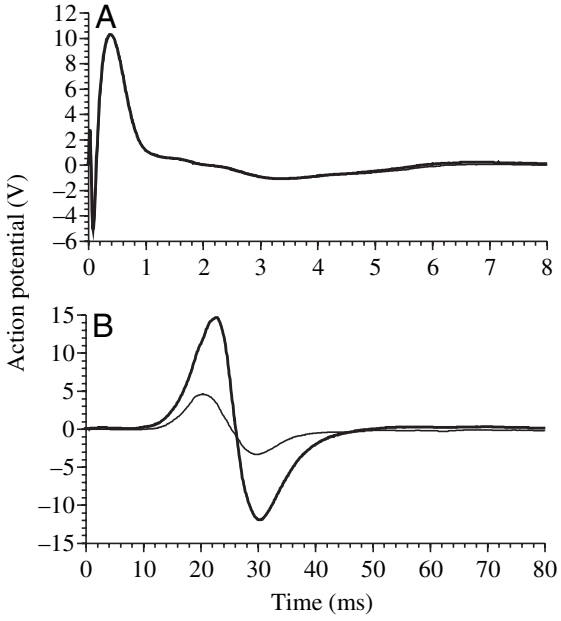

Fig. 10. Typical action potentials recorded by suction electrodes from nerve (A) and muscle (B) in a nerve–iliofibularis muscle preparation. All recordings shown are the average of eight action potentials. (A) Nerve action potentials were recorded by a bipolar lead from the nerve innervating the muscle at 4°C at the resting muscle length (thin line) and at the stretched length by 7.7% above resting length (thick line). Both lines are almost superimposed and cannot be distinguished. (B) Muscle action potentials were recorded by a monopolar lead from the surface of the muscle at 4°C at the resting muscle length (thin line) and at the stretched length (7.7% above resting length; thick line). The artifact from electrical stimulation was superimposed at the beginning of the recordings in A.

muscles with nerves, and stretch might result in an increase in the number of activated muscle fibres. This idea is also supported by the electrical activities recorded from muscle, which were augmented by a stretch, whereas action potentials recorded from nerve were the same before and after a stretch (Fig. 10). Some investigators have shown the augmentation of EPP and mEPP by stretch. Chen and Grinnell (1995, 1997) put forward the hypothesis that augmentation was induced by tension on integrins in the presynaptic membrane that was transduced mechanically into changes in the position or conformation of one or more molecules involved in neurotransmitter release. This hypothesis could also explain the enhancement of twitch force observed in the present study.

Functional importance of the phenomena in situ

In the present study, muscles were stretched from l_0 (the resting length) to $1.2\times$ l_0. The physiological range within which muscle works *in situ* is thought to be at least between 0.95 and $1.15\times$ l_0, and the variations in length that may occur in the body lie within the range of the present length change. The efficiency of neuromuscular transmission could therefore vary during the course of voluntary muscular movement and this could be of functional significance when fibres exist on the subliminal fringe of excitation. When a muscle is cooled, some muscle fibres may be just below the threshold and a stretch could evoke contraction by facilitating the transmission. In poikilothermic animals such as frogs, force enhancement by stretch may be of basic physiological importance when the temperature decreases rapidly as, if the low temperature continues, frogs adapt to the environment by thermal acclimation and can move actively. The physiological significance of this effect has already been noted (Hutter and Trautwein, 1956).

In homeothermic animals, by contrast, the efficiency of conduction in neuromuscular junction is kept constant (Ruff, 1996, 2003). The nature of the transmission is expressed by the safety factor (SF); SF=EPP/(E_{AP}–RP), where EPP is the endplate potential amplitude, RP is the resting membrane potential and E_{AP} is the threshold potential for initiating an action potential. In these animals, the high efficiency of neuromuscular transmission is maintained during muscle stretch. The safety factor for neuromuscular transmission does not change for muscles at lengths between 80% and 120% of the resting length, because the neuromuscular junction remains rigid so that the endplate membrane does not deform when the muscle fiber changes length. The change in length can be accommodated by folding and unfolding of the extrajunctional membrane, while the endplate region remains rigid. Comparing the differences in the transmission properties between the two kinds of animals, the physiological importance of the force enhancement by stretch and its thermal acclimation in poikilothermic animals are quite clear.

Effects of stretch on force in indirectly stimulated muscle

In the present study, enhancement of twitch force was not influenced by the velocity of stretch but was critically dependent on the amplitude of stretch. The enhancement appeared immediately after a stretch and was maintained. The long-lasting enhancement is reminiscent of the well-known ‘residual force enhancement after stretch’ (Edman et al., 1978, 1982; Edman and Tsuchiya, 1996; Herzog and Leonard, 2002; Herzog et al., 2003) but the effects observed in the present study are different from this, as explained below.

Force enhancement effects that have not hitherto been reported are that a muscle stretch applied during the silent period between twitches results in a big force enhancement following the stretch, and this enhancement persists as long as the muscle is elongated (Figs 4 and 7), whereas so-called ‘force enhancement following a stretch’ disappears when a muscle is deactivated (Edman et al., 1978). These differences suggest that the activation of muscle cells themselves is not directly involved in the present mechanism.

We measured the electrical signals, because the conduction of electrical signals between nerve and muscle seemed to be involved in the phenomenon. We used suction electrodes (0.35 mm and 0.8 mm i.d. tip) for nerve and muscle, respectively, to record action potential (Fig. 10). The number of nerve and muscle fibres thus measured by the electrode was approximately 17 and 8, respectively, assuming that the average diameters of a nerve and a muscle fibre are 20 μm and 100 μm, respectively. Therefore, the amplitude of the action potential may reflect the total signals from fibres measured. Yepy et al. (1974) mentioned that the increased amplitude of the action potential in a compound muscle might be due to the change in impedance of the muscle tissue upon stretch. But it may be natural to interpret the result (Fig. 10) as an increase in the number of the activated muscle fibres after stretch.

Effects of temperature on twitch force at various muscle lengths

One of the interesting results we observed is that twitch force in a nerve–muscle preparation increased by stretch at short lengths and approached the regression line of the length–force relationship measured on directly stimulated muscle as length increased, before declining along the descending limb of the relationship (Fig. 6). This effect was influenced remarkably by temperature. At high temperature (22°C), 70–80% of muscle fibres were activated at resting length (l_0) without stretch and 20–30% could be readily activated by a stretch (Fig. 6). Chen and Grinnell (1997) showed that a change in temperature had no measurable effects on the change in EPP amplitude and frequency evoked by stretch between 13°C and 22°C. Their result seems to be different from ours and the difference may arise from thermal acclimation, discussed below.

Thermal acclimation in force development

It is known that events evoked at the neuromuscular junction by an action potential of the nerve terminal, i.e. the release of neurotransmitter substance, diffusion across the synaptic gap, and the response of the end-plate on muscle, are all sensitive to temperature. Katz and Miledi (1965) showed that the decay

phase of an action potential in the nerve terminal was lengthened, and synaptic delay prolonged, at low temperatures. The amplitude of end-plate current (EPC) was reduced at cooler temperatures (Adams, 1989; Molgo and Van der Kloot, 1991). These effects can be explained by a decrease in high-threshold calcium channel activity of the nerve terminal, the reduced affinity of the receptor on the end-plate, and the reduction in the number of receptor channels available at low temperatures (Janssen, 1992). Thermal acclimation at the nerve–muscle junction is possible in any part of the pathways above-mentioned, but only a few effects have been reported so far. The resistance of neuromuscular transmission to cold in the sartorius muscle was increased when frogs were kept at low temperatures for prolonged periods (Jensen, 1972) and the latency of the leg withdrawal reflex was shortened by cold acclimation (Tiiska and Lagerspetz, 1999).

In the present study, we measured the twitch force and its enhancement by stretch at 4°C after the frogs had been kept at 4°C for more than 2 months and found that high twitch force was induced at the resting length (l_0) (Figs 8 and 9). Thus, the present experiment clearly adds another example of thermal acclimation, based on our experience in the present study. On transfer from room temperature to a low temperature in the laboratory, immediately after the transfer frogs crawled very slowly, but their behavior gradually livened up and after a few months at the low temperature they could jump. Previous studies, by contrast, reported that the mechanical performance of frog muscle was not different after long-term exposure to different temperatures (Renaud and Stevens, 1984; Rome, 1983). Thermal acclimation of locomotory performance in frog is still a matter of controversy (Brattstrom and Lawrence, 1962; Renaud and Stevens, 1983; Wilson and Franklin, 2000; Wilson et al., 2000). The frogs *Rana brevipoda* in the present study in Japan are active in a warm environment, i.e. 14–27°C from April to October, and in hibernation for the remaining months, which is different from the behaviour of the frogs *Rana temporaria* and *Rana pipiens* used commonly in Europe and America. Large variations in the sensitivity of thermal acclimation may exist in animals in different geographic habitats (Miller and Dehlinger, 1969; Wilson, 2001).

Effects of temperature on twitch force

Our result showed that twitch force following indirect stimulation was lower at lower temperatures (Fig. 3). The magnitude of twitch force in a whole muscle is the total amount of twitch force produced by all fibres, and the number of recruited muscle fibres for activation is smaller at lower temperature. There are several possible reasons for this. (1) The decrease in twitch force at low temperature is caused by the reduction in excitability of nerve that innervates a muscle. This is not provable, however, because full tetanic force was induced by indirect stimulation at low temperatures (Figs 2 and 3). (2) The reduction in the efficiency of conduction at the neuromuscular junction at low temperature. Previous investigators (Foldes et al., 1978; Harris and Leach, 1968) reported that the decrease in both release of acetylcholine (ACh) and activity of acetyl cholinesterase (AChE) occurred together at low temperatures, but the extent of the decrease in AChE activity was higher than that of ACh release, resulting in a net decrease in the available ACh. Adams (1989) reported that the proportion of fibres producing subthreshold EPC in response to a single nerve shock was 42% at 5°C and 59% at 2.5°C. The proportion that we found was approximately 88% at 4°C (Fig. 3). Thus our results, together with those of the previous report, suggest that temperature strongly affects transmission at the neuromuscular junction.

Authors express their hearty thanks to Profs H. Kitagawa, Kobe University, A. Matsuno, Shimane University and M. Nakamura, Waseda University, for their invaluable advice in the course of the experiment. The authors are also grateful to Mr H. Itoh for his effectual technical assistance.

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