

# Nonlinear Summation of Contractions in Cat Muscles

## *II. Later Facilitation and Stiffness Changes*

F. PARMIGGIANI and R. B. STEIN

From the Department of Physiology, University of Alberta, Edmonton, Canada T6G 2H7. F. Parmiggiani's permanent address is C.N.R. Laboratory of Central Nervous System Physiology, Milan, Italy.

**ABSTRACT** The force produced by cat muscles over time with two stimuli separated by a short interval is approximately three times that produced by a twitch of cat muscles. This facilitation of force production by a second stimulus involves both increases in magnitude and duration of the contraction. Increased magnitude is relatively more important in the fast-twitch plantaris muscle, whereas increased duration is more important in the slow-twitch soleus muscle. The facilitation decays in an approximately exponential manner with the interval between stimuli, having a time constant between one and two times the twitch contraction time in different muscles. If a third stimulus is added, the greatest facilitation is seen at intervals longer than the twitch contraction time. The drug Dantrolene, which specifically reduces  $\text{Ca}^{++}$  release from the sarcoplasmic reticulum, eliminates the delayed peak in facilitation with three stimuli. Associated with the increases in force with one or more stimuli are increases in muscle stiffness, which can be measured with small, brief stretches and releases that do not alter the time-course of contraction. The stiffness of soleus muscle reaches a peak after the peak in force. The increasing stiffness of the muscle can considerably facilitate transmission of force generated internally, in addition to any facilitation arising from  $\text{Ca}^{++}$ -release mechanisms.

### INTRODUCTION

If a second stimulus is superimposed on the rising phase of a twitch, a less-than-linear summation of force generation ("depression") is initially observed (Stein and Parmiggiani, 1981). At about the peak of the twitch, this early depression gives way to a more-than-linear summation or later "facilitation." This paper analyzes the nature and time-course of this facilitation in detail. Several possible mechanisms can be imagined for the facilitation of force production by successive stimuli. For example, a facilitated release of  $\text{Ca}^{++}$  from the sarcoplasmic reticulum might occur—similar to the well-known facilitation of transmitter release at the neuromuscular junction (Mallart and Martin, 1967). However, the evidence for a facilitation of  $\text{Ca}^{++}$  release is

equivocal (Blinks et al., 1978; Desmedt and Hainaut, 1978). Even a reduced release of additional  $\text{Ca}^{++}$  by a second stimulus might facilitate production of tension through a cooperative interaction with  $\text{Ca}^{++}$  released by the first stimulus if more than one  $\text{Ca}^{++}$  ion must bind to troponin before force is produced (Hartshorne and Pyun, 1971; Ashley, 1978).

Facilitation could also arise from interaction between the contractile elements in the muscle and elastic elements such as tendons in series with them. To understand how this could occur, consider an elastic band which is held at less than slack length. The first stretch applied to the elastic band will merely take up the slack without producing force. Second and subsequent stretches would then produce increments in force. The "facilitation" of force output after the first stretch results from the nonlinear behavior of the elastic band below slack length. However, the elastic elements in muscle are nonlinear over their entire range, showing a monotonically increasing stiffness with increasing muscle length.

Muscles also contain viscous elements that, in principle, could contribute to the later facilitation. During the falling phase of an isometric twitch, the series elastic elements will shorten as the tension falls in the contractile elements. The shortening of the series elastic elements will stretch out the contractile elements toward their relaxed length. A muscle that is being stretched while it is trying to shorten can generate more than its isometric force (Katz, 1939).

To decide among possible explanations for the later facilitation, two types of experiments were carried out. First, the time-course of the facilitation was studied in more detail before and after application of the drug Dantrolene, which reduces  $\text{Ca}^{++}$  release. Second, the changes in stiffness that accompany twitches or short trains of stimuli were studied and compared in time-course to the facilitation. In addition to any stiffness changes taking place in passive elastic elements, a muscle becomes stiffer as a result of the increased number of cross-bridges formed between actin and myosin during force generation. Ford et al. (1977 and 1981) have studied in detail the forces generated in response to small, fast stretches of isolated, single frog muscle fibers. These changes in force represent changes in muscle stiffness, since stiffness is merely the change in force per unit change in length. Under their conditions, most of the stiffness arose directly from the bonds between the thick and thin filaments of the contracting muscle, rather than from tendons or other passive structures.

Although stiffness changes have been studied during tetanic contractions of mammalian muscles (Joyce et al., 1969; Morgan, 1977), there is only a brief report of changes occurring during a twitch or other brief contraction. To the extent that stiffness changes can be measured and related to the number of bonds formed between contractile proteins, one can assess whether the facilitation is associated with increased bond formation within the muscle or merely improved transmission of force to external recording devices. The results suggest that a prolonged release of  $\text{Ca}^{++}$  and improved transmission of force by a stiffer muscle both contribute to the facilitation observed in whole mammalian muscles.

## METHODS

Most of the data in this paper are from the same experiments as in the accompanying paper (Stein and Parmiggiani, 1981) and details of some methods used are given in that paper. In addition, stiffness changes occurring in cat muscles were measured by applying small stretches to the muscles with frequencies varying from 10 to 100 Hz. The modulation in stiffness was not as great with the highest frequency stretches and was not observed when stretches were applied at  $>100$  Hz. However, with these rapid stretches, the mass (4.6 g) of the force transducer provided some inertia and the stretch may not have been adequately transmitted to the tendon of the muscle. Some mass is necessary for a muscle of this size generating tens of Newtons, so the lack of modulation in stiffness at very high frequencies may be a result of technical inadequacies of the preparation.

A torque motor (TQ40W; Aeroflex Laboratories Inc., Plainview, N. Y.) was used with feedback circuitry (designed by Dr. S. Andreassen) so that it had an effective stiffness of 50 N/mm. Thus, maximal twitch contractions of whole cat muscle could be recorded under nearly isometric conditions. The stretch and release phases of the square-wave length changes were controlled by digital circuitry so that the phase of the stimulus with respect to the stretch could be varied (see Fig. 1). Typically, five sweeps each were averaged for stimuli applied at phases of 0, 90, 180, and 270° with respect to the square wave stretch, and data were stored directly in a digital computer (PDP 11-34; Digital Equipment Corp., Maynard, Mass.).

Fig. 1A shows the superimposed forces produced during a twitch with stretches applied 180° out of phase and the average of these two stretches (*smooth, central curve*), which cancels out the effect of the stretch. By taking the difference of the two *outer curves*, the force produced by the stretch can be seen in isolation (Fig. 1B). Peak-to-peak measurements were made (Fig. 1B, *arrows*) for the stretch-induced tension and divided by the corresponding length changes (Fig. 1D) to obtain stiffness values at various times during the twitch (Fig. 1C). The values were then connected by straight line segments to give a smooth curve of stiffness with time that could be compared with the force in Fig. 1A.

## RESULTS

*Two Stimuli*

The *upper half* of Fig. 2 shows the pattern of summation produced when a second stimulus is applied at different times relative to the first for a slow (soleus) and fast (plantaris) muscle. Both muscles show a similar pattern in which the response to two stimuli at short intervals is much greater than expected for linear summation of twitch responses, as was first pointed out for cat muscles by Cooper and Eccles (1930; see also Ranatunga [1978]). The extent of this facilitation is seen more clearly when the contributions of the second stimulus to the total force production are compared with the twitch responses. The facilitation tended to be largest with short intervals, and the area contributed by the second stimulus (integral of force over time) was  $213 \pm 36\%$  (mean  $\pm$  SD) for 10 soleus muscles and  $207 \pm 71\%$  for 10 plantaris muscles. Thus, the area contributed by the second stimulus was more than twice that of the first or the force integrated over time in response to two stimuli was more than three times that of the twitch. Burke et al. (1976)

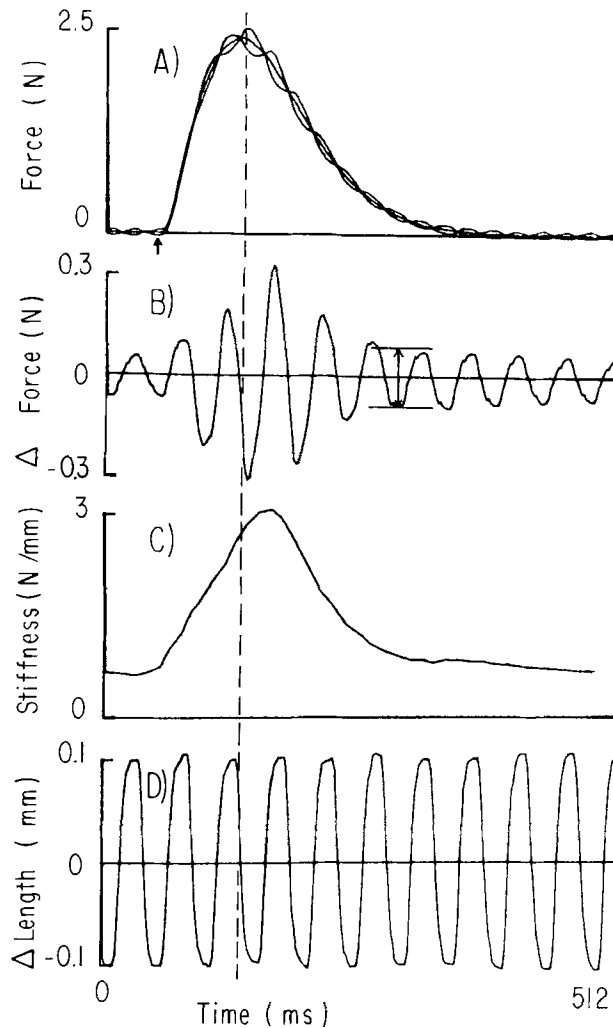


FIGURE 1. (A) Twitch force produced by a maximal stimulus (arrow) to the nerve of soleus muscle in the cat during application of alternating stretches and releases at two opposite phases (outer curves) and the mean of these two (central curve). (B) The difference in tension resulting from the stretch at the two phases has been magnified to show the increasing tension produced by each cycle of the stretch during the twitch contraction. The peak-to-peak difference in tension was measured from each peak to the mean value of the two nearest peaks, as indicated by the double-headed arrow. (C) The stiffness of a muscle is the ratio of the force produced per unit change in length, and is related to the number of bonds formed between actin and myosin in muscle. Values of stiffness were computed from the peak-to-peak differences in force in (B) and from stretches at other phases relative to the twitch. Measured points were then joined by straight line segments. Note that the maximum stiffness occurred after the peak in force (vertical dashed line). (D) The difference in length produced by stretches with opposite phase was calculated as in (B) above, which gives twice the value of the length changes during any one cycle. A relatively low frequency (20 Hz) is shown in this figure for purposes of illustration, but similar results were obtained with a range of frequencies up to 100 Hz.

observed an even greater facilitation for single motor units from the cat medial gastrocnemius muscle. For the sixteen motor units we studied from cat soleus muscle, the area contributed by the second stimulus was  $319 \pm 70\%$  that of the twitch, which was significantly  $>213\%$  indicated above for the whole muscles.

The increased area contributed by the second stimulus arises from a greatly increased time-course of tension generation as well as an increase in amplitude. The peak in the tension contributed by the second stimulus in the cat soleus muscles occurred at a time  $87 \pm 19\%$  greater than the contraction time for the twitch. The peak for a second stimulus to cat plantaris muscle was reached at a time  $37 \pm 18\%$  greater than the contraction time for the twitch. Contraction times were measured from the stimulus rather than from the EMG or the

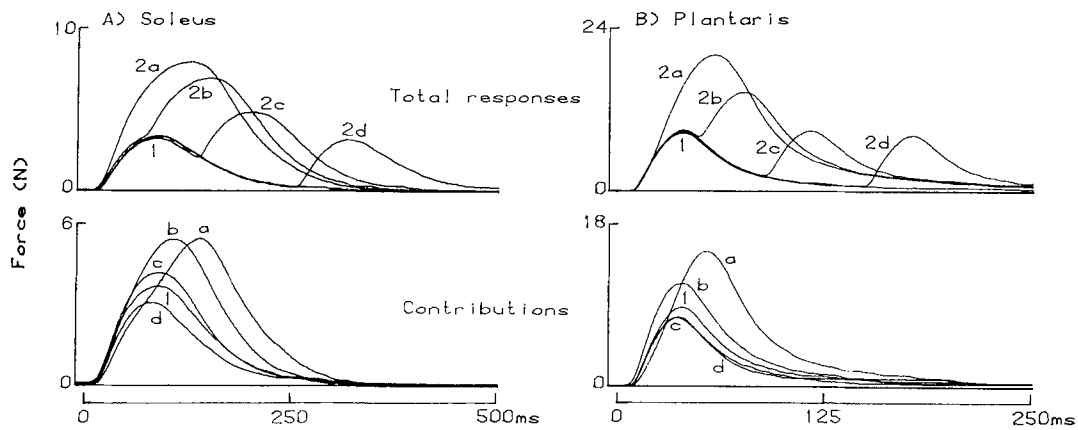
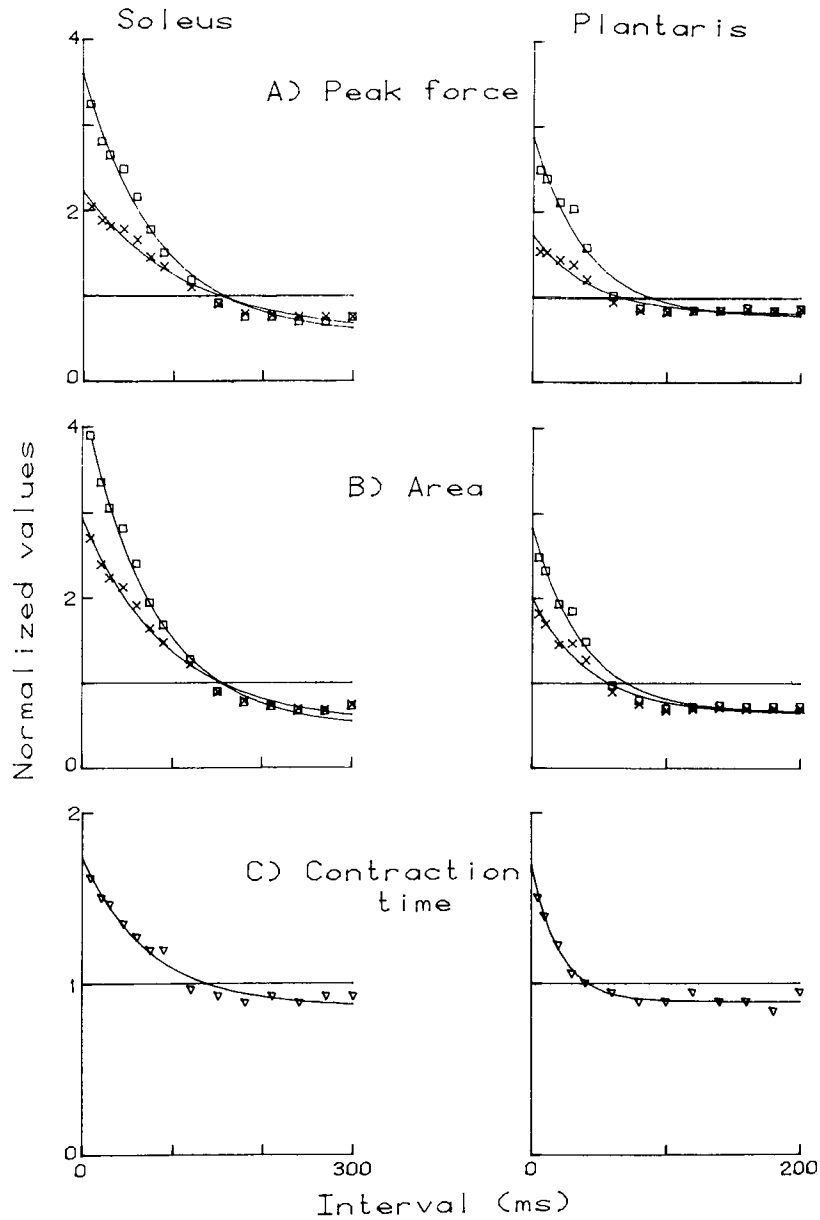


FIGURE 2. Superimposed contractions resulting from one stimulus (twitch) and two stimuli at a variety of intervals (2a-2b) for a slow-twitch (soleus) muscle (A) and a fast-twitch (plantaris) muscle (B). The force contributed by each stimulus was computed as in the accompanying paper (Stein and Parmiggiani, 1981) and compared with the twitch for the intervals shown. Note that the force contributed by the second stimulus was largest and slowest in time-course for the shortest intervals and declined to less than the twitch (1) for long intervals.

onset of contraction. If delays in conduction, neuromuscular transmission, and excitation-contraction coupling were subtracted, the percentage slowing would be even greater. Although both muscles showed a progressive slowing of the responses to later stimuli, the slowing was not as great for plantaris muscle, even relative to its briefer contraction time. Thus, the similar degree of facilitation was produced in somewhat different ways in the two muscles: slowing of the already slow soleus muscle accounted for more of the facilitation in that muscle, whereas the increase in the peak tension was greater in the plantaris muscle.

The area, peak tension, and time-to-peak force are plotted in Fig. 3 for the second stimulus relative to the values for the twitch. Note that the magnitudes of all three parameters decrease with the interval between stimuli in a smooth, approximately exponential fashion and tend to undershoot the horizontal line

$y = 1$  (the value for the twitch). The undershoot represents a slow depression that will be considered elsewhere. Particularly at short intervals, the values could be affected by the early depression, which was analyzed in the accompanying paper (Stein and Parmiggiani, 1981). We showed that this depression could arise from a saturating, first-order reaction. A transformation was introduced (Eq. 5 in Stein and Parmiggiani [1981]), which provided a mathematical basis for the separation of early depression from the later



facilitation. The corresponding values after using this transformation are shown (*open squares*) in Fig. 3. They are somewhat larger, but the exact values will depend, for example, on the implicit assumption in the transformation that the saturation reaction is independent of the later facilitation. Note, however, that the time-course of the facilitation is much the same before and after the transformation and is therefore unlikely to depend greatly on the assumptions used. The time-course is also similar for the fast- and slow-twitch muscles shown in this figure when intervals ( $t$ ) are plotted relative to their different contraction times.

Each set of data points in Fig. 3 was fitted with a curve of the form  $y = a + b \exp(-t/\tau)$  according to a least-mean-squares criterion. These fitted curves are also plotted in Fig. 3 and the values of  $\tau$  were generally between one and two times the twitch contraction time for both muscles and for all parameters (changes in peak, area, and contraction time) both before and after the transformation. This suggests that the processes that determine the contraction time of a twitch may also determine the facilitation of a second stimulus.

### *Three Stimuli*

Fig. 4 shows the response of soleus and plantaris muscles to a third stimulus applied at various times after two stimuli with a short interval (10 ms). If the third stimulus is also given at a short interval, the early depression is more marked and the later facilitation is less prominent (Stein and Parmiggiani, 1979*b* and 1981). There is still some net facilitation, and this facilitation becomes greater at longer intervals because of the difference in time-course between the early depression and the later facilitation. Fig. 5 gives the parameters for the contribution of the third stimulus as a function of the interval between the second and third stimulus. The optimal interval for contributing additional area or peak tension was  $1.50 \pm 0.20$  (mean  $\pm$  SD) times the twitch contraction time in soleus muscle, and  $1.26 \pm 0.15$  times the contraction time in plantaris muscle. Thus, the optimal pattern for activating mammalian muscles consists of a doublet (i.e., a short interval) followed by a longer interval (Stein and Parmiggiani, 1979*b*; Zajac and Young, 1980).

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FIGURE 3. (Opposite). The peak force ( $A$ ), the area under the curve of force versus time ( $B$ ), and the time for the contraction to reach its peak ( $C$ ) were computed for the force contributed by the second in a pair of stimuli at a variety of intervals for cat soleus and plantaris muscles (see examples in Fig. 2). Values in ( $A$ ) and ( $B$ ) are shown before ( $\times$ ) and after ( $\square$ ) using the transformation of Eq. 5 in Stein and Parmiggiani (1981) to eliminate the saturation process underlying the early depression. The facilitation is increased but its time-course is not greatly changed by the transformation. Therefore, the contraction times ( $\nabla$ ) are not shown after a transformation. Exponential curves were fitted using a nonlinear, least-mean-squares algorithm (Hartley, 1961) and superimposed on the data. All values are measured relative to that of the twitch (*horizontal line* with a value of 1). Values  $>1$  represent a net facilitation ( $A$  and  $B$ ) or slowing of contraction ( $C$ ); values  $<1$  represent a net depression or speeding up of the time-course of the force contributed by the second stimulus.

In contrast, the contraction time of the contribution produced by the third stimulus was greatest at short intervals, and decreased smoothly with increasing intervals. The contraction time for the third contribution was also greater than for the second (see Fig. 3) and the time constants of the fitted exponential curves were generally longer than for the second stimulus.

The tendency for the optimal interval for force production to occur with rather long intervals might arise because the force is quite high at earlier times and the saturating reaction is limiting the degree of facilitation possible. Again, Eq. 5 of Stein and Parmiggiani (1981) could be used under the assumption that the early depression arose from a first-order, saturating reaction. After this transformation (Fig. 5, *open squares*), the facilitation at early times is considerably greater, but a delayed peak is still evident.

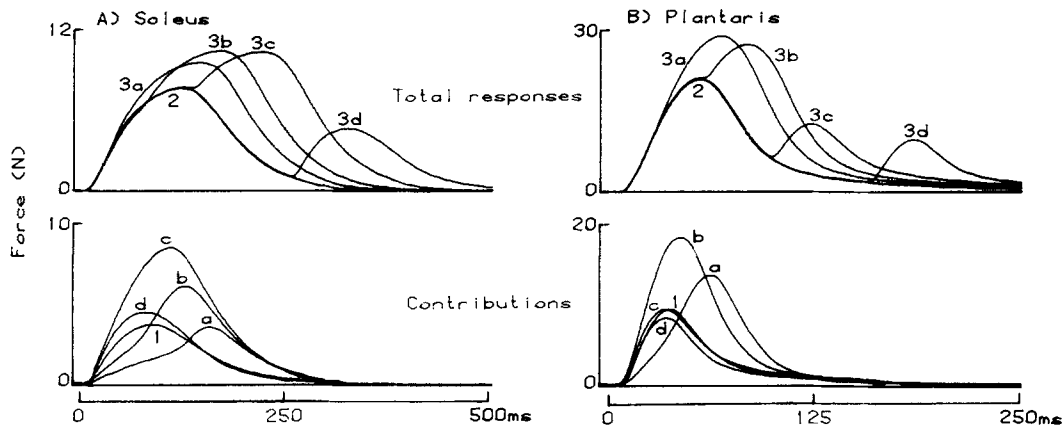


FIGURE 4. Superimposed contractions for two stimuli at a short interval (10 ms) and a third stimulus at a variety of intervals (3a-3d) for soleus (A) and plantaris (B) muscle. The force contributed by the third stimulus was computed as in Fig. 2 and compared with that produced by the first stimulus (twitch). The contribution of the third stimulus is largest at intervals that are longer than the contraction time for both muscles. See text for further discussion.

Occasionally, a plateau or even a delayed peak was seen in the contribution of the second stimulus. A plateau or delayed peak in the facilitation produced by a second stimulus tended to occur under conditions where the twitch-to-tetanus ratio was large. A possible role of  $\text{Ca}^{++}$  release as a mechanism for facilitation was examined by using Dantrolene, which reduces the twitch by ~50% by reducing  $\text{Ca}^{++}$  release.

#### *Dantrolene*

Dantrolene abolishes the early depression for the second or even the third pulse (Stein and Parmiggiani, 1981). The facilitation and the increase in time to peak produced by this drug are shown in Fig. 6. The magnitude of the facilitation was greatly increased (relative to the much smaller twitch) and its time-course was shortened (even relative to the faster contraction time of the



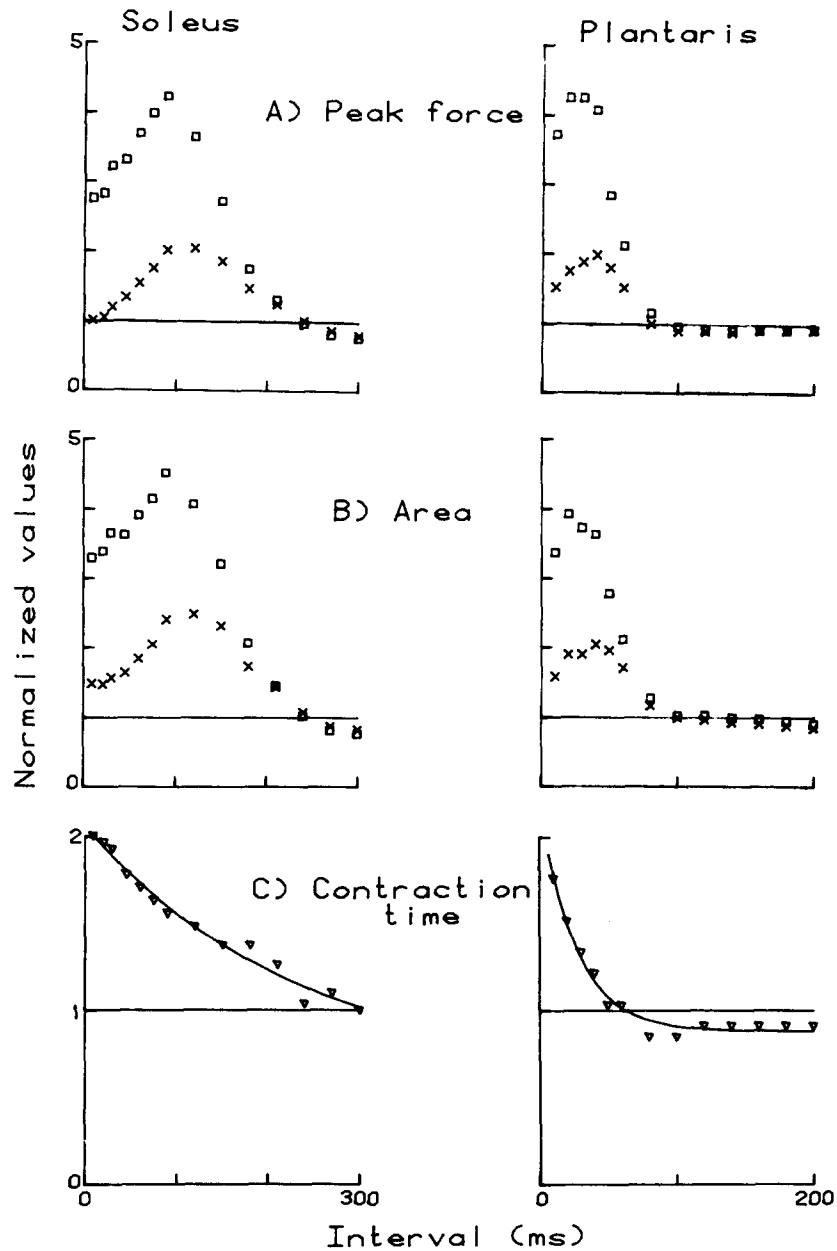


FIGURE 5. The peak force (A), the area under the curve of force versus time (B), and contraction time (C) were computed as in Fig. 3 for the force contributed by the third stimulus in a train at a variety of intervals for cat soleus and plantaris muscles. In each case, the first two stimuli in the train were given at a short interval (9-10 ms). Values are all measured relative to the values for a single stimulus (twitch) and are shown before (X) and after (□) being transformed, as in Fig. 3. A delayed maximum is observed in the peak force and the area contributed by the third stimulus, but not the contraction time, which follows a simple exponential curve. See text for further discussion.

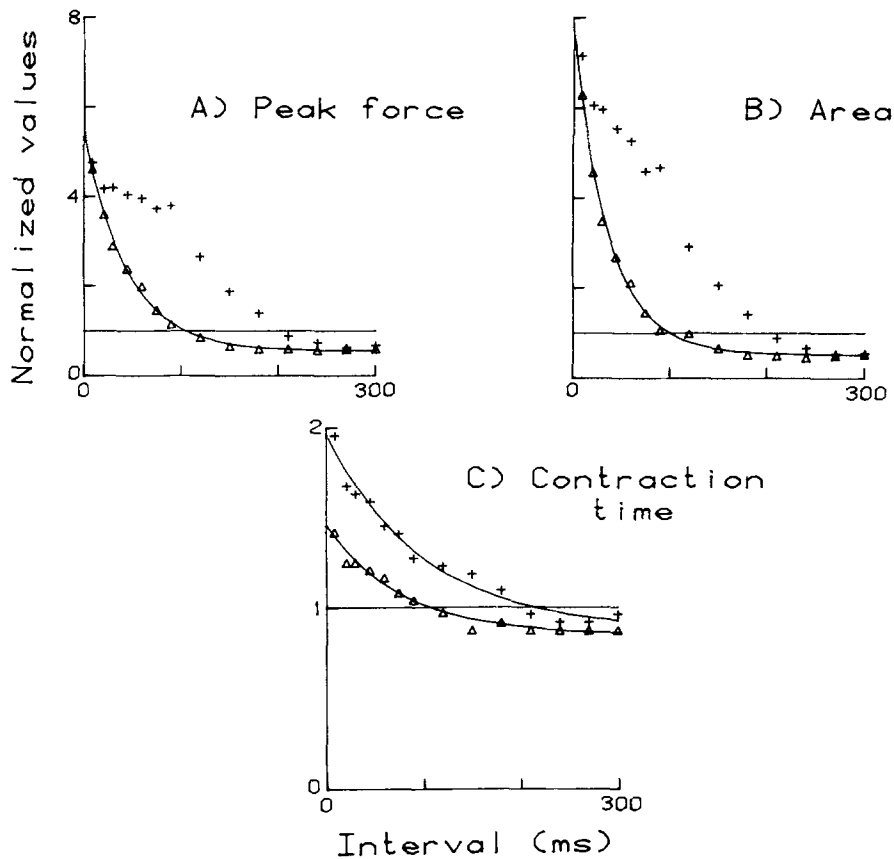


FIGURE 6. Dantrolene reduces the magnitude of the twitch to one-half or less of original magnitude and shortens its time-course somewhat. However, the facilitation of peak force (A) and the area (B) contributed by the second ( $\Delta$ ) and the third (+) stimuli are increased relative to the smaller twitch contraction (horizontal lines at  $y = 1$ ). Compare with Fig. 3 for two stimuli and Fig. 5 for three stimuli given to untreated cat soleus muscles. Note that the delayed maximum in Fig. 5 for the facilitation with three stimuli has been eliminated, but there is a hint of a plateau. Data have been fitted with exponential curves using a nonlinear, least-mean-squares algorithm where appropriate.

twitch). The delayed peak observed with three pulses before Dantrolene is no longer evident, although there is a hint of a plateau.

#### *Stiffness*

Small stretches at various rates could be superimposed on an ongoing contraction, as described in the Methods. The stretches were small enough ( $\sim 0.1\%$  of muscle length) that they should provide a measure of the number of bonds between actin and myosin (Flitney and Hirst, 1978) without disrupting the time-course of the contraction.

We consistently found for cat soleus muscle (Fig. 1A; see also Stein and

Parmiggiani [1979*a*]) that the peak in stiffness occurred after the peak in twitch tension. An even greater delay was observed with longer trains of stimuli, as shown in Fig. 7. The mean delays in peak stiffness compared with the peak in tension were: 1 stimulus,  $24.7 \pm 8.0$  ms (mean  $\pm$  SD,  $n = 18$ ); 2 stimuli,  $32.5 \pm 14.7$  ms ( $n = 8$ ); 3 stimuli,  $47.0 \pm 5.3$  ms ( $n = 6$ ); 4 stimuli,  $46.0 \pm 4.6$  ms ( $n = 6$ ); 10 stimuli,  $4.18 \pm 4.6$  ms ( $n = 12$ ). In contrast, the fast-twitch cat plantaris muscle consistently showed a peak in stiffness before the maximum tension, although there might be a secondary peak after the maximum tension (Stein and Parmiggiani, 1979*a*).

A subtraction procedure could be used to measure the contribution of each stimulus to the changes in stiffness, as well as the force production by the

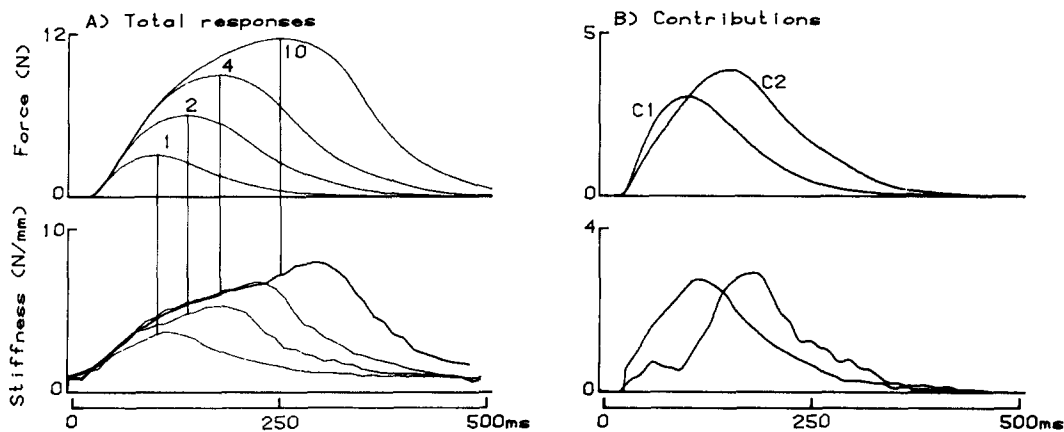


FIGURE 7. (A) Comparison of the force (*upper curves*) and stiffness (*lower curves*) produced by various numbers of stimuli to cat soleus muscle, as indicated in the *upper part* of this figure. Vertical lines are drawn from the peak in the contraction for comparison with the peak of stiffness, which occurs in each case at a later time than the maximal force. Stiffness was calculated as indicated in Fig. 1. (B) Contributions of the first two stimuli to the total force and stiffness produced. Although there is substantial facilitation in the force contributed by the second stimulus, there is virtually no increase in the peak of stiffness and a slight net decrease in the area contributed by the second stimulus to the total stiffness.

muscle. On the right of Fig. 7 are shown the contributions of the first two stimuli. The contribution of the second stimulus to the force shows a small early depression followed by a larger late facilitation, so that there was a large, net facilitation under these conditions. However, there is no net facilitation of stiffness. Stiffness changes are so markedly depressed soon after the second stimulus that this depression more than offsets the later facilitation of stiffness that is seen. This lack of net facilitation in stiffness to a second stimulus was consistently observed in all experiments in which force and stiffness were simultaneously measured.

In several experiments we measured the effect of changing muscle length on the resting stiffness and the peak additional stiffness during a twitch. As

shown in Fig. 8 the resting stiffness (before stimulation) increased monotonically with muscle length. The extra stiffness during the twitch increased and then decreased with length roughly in parallel with the active tension (Fig. 8). The variation in active tension depends on the overlap between thick and

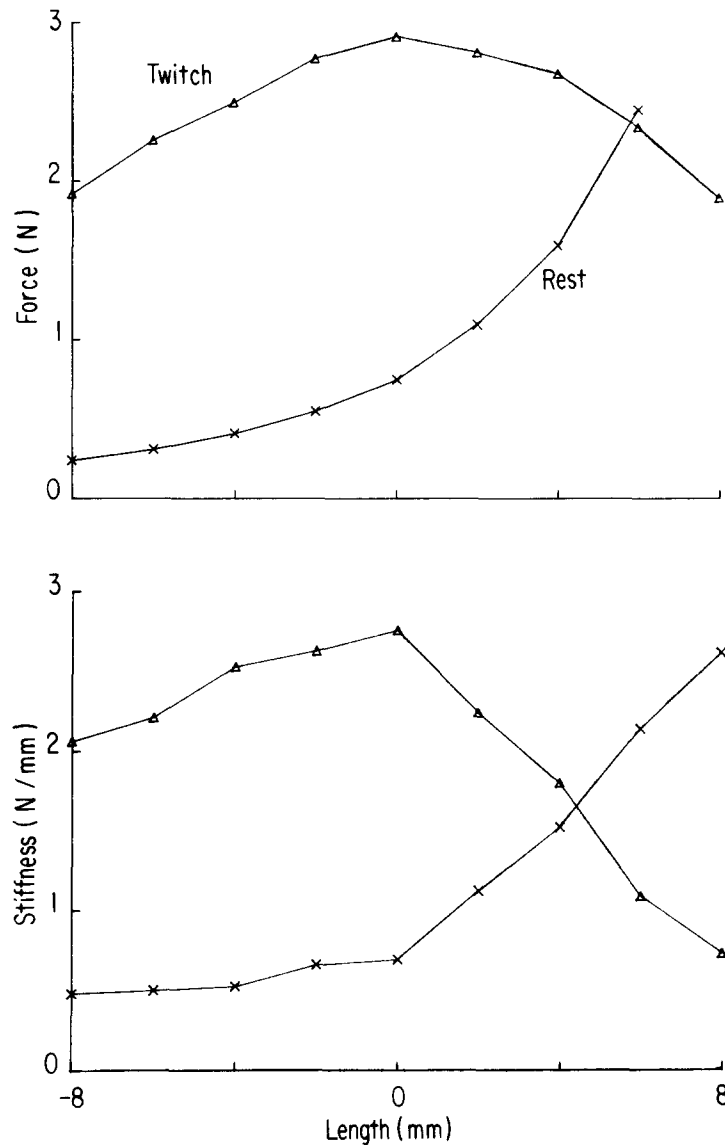


FIGURE 8. Comparison of the force (*upper curves*) and stiffness (*lower curves*) produced by a soleus muscle at rest ( $\times$ ) and during a twitch contraction ( $\Delta$ ) at various lengths. The force and stiffness shown for the twitch give the peaks measured after subtracting the resting values. Length was measured relative to the length which gave the maximum twitch tension.

thin filaments (Gordon et al., 1966), and hence the number of bonds that can be formed between actin and myosin. The parallel variation in stiffness supports the view that the measured stiffness also depends on bond formation. These results and their relation to the facilitation of tension production will now be discussed.

#### DISCUSSION

One stimulus markedly facilitates the tension produced by a second stimulus (Burke et al., 1976; Ranatunga, 1978; Zajac and Young, 1980). Two stimuli to a cat muscle at body temperature often produce more than three times the tension integrated over time that a single stimulus does, instead of a doubling that would be expected from linear summation. The increased tension-time integral depends to different degrees in fast- and slow-twitch muscles on increases in the peak tension and prolongation in tension production, but both processes contribute to the facilitation in each type of muscle. Although aspects of this facilitation were described over 50 years ago (Cooper and Eccles, 1930), there are still few clues to its cellular and molecular basis. In principle, the facilitation could arise at any of the stages involved in excitation-contraction coupling (Introduction), but our experiments introduce some constraints on the mechanisms that might underlie facilitation. Individual results will be discussed briefly before outlining what we feel is the most likely sequence of events.

The facilitation of the tension produced by a second impulse decayed in a simple exponential fashion with the interval between stimuli. This was true whether the facilitation was measured in terms of the peak tension contributed by the second stimulus, the increase in the time to peak or the area under the curve (the tension-time integral). In comparing fast- and slow-twitch muscles, the time-course of the facilitation was closely related to the contraction time of the muscle. Thus, the same processes that determine the contraction time of the twitch are likely to be involved in producing the facilitation of tension by a second impulse.

The pattern of facilitation is markedly different for the third and later stimuli. Whereas the facilitation to the second pulse decays as a simple exponential function of the interval between stimuli, the optimal interval for facilitation of the third stimulus is  $>100$  ms in slow-twitch muscles. Again, comparing different muscles, the optimal interval is closely related to the contraction time. The optimal interval between stimuli was 1.26 and 1.5 times the contraction time of the twitch in the fast- and slow-twitch muscles studied. Burke et al. (1976) studied motor units with a wide range of contraction times and found that the optimal interval was generally 1.3–1.4 times the twitch contraction time. Thus, again there appears to be a correlation between the mechanisms that determine the contraction time and those that affect the facilitation of tension production.

#### *Stiffness*

The first detailed measurements of stiffness changes during a twitch or a short train of impulses are given in this paper. Earlier work on frog muscles

(Buchthal and Kaiser, 1944; Julian and Sollins, 1975) indicated that stiffness changes more or less in parallel with the production of tension. If the changes in stiffness represent alterations in the number of bonds formed between actin and myosin (Flitney and Hirst, 1978; Ford et al., 1977), the peak in stiffness should precede the peak in tension somewhat since the viscoelastic lags in transmission of force to the tendon can only produce delays in the recorded tension.

Stiffness changes that precede force changes were, in fact, recorded for fast-twitch mammalian muscles but not for slow-twitch muscles (Stein and Parmiggiani, 1979*a*). The delay in reaching the peak of stiffness increased with the number of stimuli (Fig. 7), which suggests that stiffness depends on the type as well as the number of bonds formed. We have argued elsewhere (Stein and Parmiggiani, 1979*a*) that late in the time-course of the contraction, more of the bonds in slow-twitch muscle could be in a different state; e.g., the *rigor* state (Weber and Murray, 1973). In this state, bonds can generate considerable stiffness at relatively low force levels (White, 1970; Kawai and Brandt, 1977).

Whatever the mechanism may be for the increased stiffness during the twitch, this increase could also be responsible for much of the facilitation of the second stimulus by improving transmission of the force generated internally to an external recording device (see below). Indeed, if the stiffness measurements give an accurate indication of the number of bonds formed between actin and myosin, and if the second stimulus produces no net facilitation of extra stiffness while producing a large net facilitation in force production (Fig. 7*B*), then improved transmission of internal force via the viscoelastic properties of the muscle is the *only* means for producing a net facilitation of force production.

This striking conclusion rests on the assumption that the stiffness measurements do provide an indication of actomyosin interaction. Since we used a large mammalian muscle at body temperature, our time resolution was limited and some turnover as well as deformation of bonds may have occurred. However, there are several reasons for confidence in the stiffness measurements; (*a*) Turnover rates for actomyosin ATPase, even under optimal conditions *in vivo*, are quite low. For example Hartshorne et al. (1972) measured rates of 1–1.5 mol P/min·mg protein for rabbit muscle myofibrils at 37°C, which corresponds to turnover rates of  $\sim 10\text{ s}^{-1}$  for each myosin head (Marston and Taylor, 1980). This value is comparable to the rate constants for the exponential increase in tension during a tetanus in fast- ( $\sim 20\text{ s}^{-1}$ ) and slow-twitch ( $\sim 10\text{ s}^{-1}$ ) cat muscles (R. B. Stein and F. Parmiggiani, unpublished observations). (*b*) We obtained qualitatively similar results using a range of frequencies from 10 to 100 Hz. For example, the peak in stiffness was consistently observed to occur in soleus muscle after the peak of tension at all frequencies. Turnover of bonds occurring at a fixed rate should be less and less important at higher frequencies. (*c*) Qualitatively similar results have also been obtained with mouse muscles *in vitro* (T. Gordon, R. B. Stein, and A. Thomson, unpublished observations) using frequencies up to 1,000 Hz at temperatures down to 8°C. Turnover rates are very much lower at such low

temperatures and should be even less significant at the much higher frequencies possible with the small mouse muscles. (d) The series stiffness varied roughly in parallel with force over a range of muscle lengths (Fig. 8). The variation of force with length is well known to depend on the overlap between thick and thin filaments, and hence on the number of actomyosin bonds which can be formed (Gordon et al., 1966). The parallel variation of stiffness changes during contractions implies that they also depend on the number of bonds formed.

If the stiffness measurements are accurate, what is the most likely sequence by which one stimulus can markedly facilitate the force production by later stimuli?

#### *Sequence of Events Producing Facilitation*

(a) The first stimulus in both fast- and slow-twitch muscles produces changes in the series stiffness that facilitate the transmission of force produced internally by subsequent stimuli. This process is analogous to the improved transmission of force in a taut elastic band relative to a slack one. The facilitation will be maximal at short time intervals where the force production by the second stimulus will coincide with the period of increased stiffness produced by the first stimulus. This facilitation decays exponentially with increasing intervals according to a time constant between one and two times the twitch contraction time.

(b) Force production by a second stimulus is greatly prolonged relative to the twitch. Since this prolongation is also observed in the stiffness changes (Fig. 7), it should arise from a process up to or including the formation of actomyosin bonds. A prolonged release of  $\text{Ca}^{++}$  would be a possible mechanism. A cooperative binding of  $\text{Ca}^{++}$  ions by troponin (Ashley, 1978) is unlikely to be important, since this would tend to speed rather than delay force production by late stimuli (R. B. Stein, unpublished calculations).

(c) In addition, delayed changes in stiffness occur that are specific to slow-twitch muscles. These may arise from an increased number of rigor bonds (Stein and Parmiggiani, 1979a) late in the contractions produced by one or a few stimuli. The delayed changes in stiffness may be significant for the maintenance of posture by slow-twitch muscles, since a stiff muscle will resist with considerable force any attempts to stretch it (e.g., under the force of gravity).

(d) Finally, with three or more pulses, a late peak of facilitation occurs such that the optimal interval for force production is greater than the twitch contraction time of the muscle. Some delay is required so that force does not build up to levels where saturation becomes important, but this does not appear to be the whole explanation (Fig. 5). If  $\text{Ca}^{++}$  levels reach a sufficient concentration, they can release further  $\text{Ca}^{++}$  from the sarcoplasmic reticulum ( $\text{Ca}^{++}$ -induced  $\text{Ca}^{++}$  release; see Endo [1977]) with a delay of  $\sim 200$  ms in frog muscles at room temperature (Potreau and Raymond, 1980). Interestingly, Dantrolene, which reduces  $\text{Ca}^{++}$  release from the sarcoplasmic reticulum, also eliminated the delayed peak with three pulses. The results from the

Dantrolene experiments are consistent with the involvement of  $\text{Ca}^{++}$ -induced  $\text{Ca}^{++}$  release in the delayed facilitation produced by three or more stimuli, but direct measurements of free  $\text{Ca}^{++}$  under comparable conditions in mammalian muscles (Eusebi et al., 1980) would clearly be necessary to prove this suggestion. Whatever the mechanisms of facilitation prove to be, the nonlinear summation of muscle contractions has a functional significance in the control of movement, and motoneurons appear to have adapted for effective use of these properties (Stein and Parmiggiani, 1979 *b*).

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