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FORCE–VELOCITY RELATION IN DEUTERIUM OXIDE-TREATED FROG SINGLE MUSCLE FIBRES DURING THE RISE OF TENSION IN AN ISOMETRIC TETANUS

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SUMMARY

1. The force–velocity ($P-V$) relation for single fibres isolated from the semitendinosus muscle of the frog was determined at pre-set times during the rise of tension and the plateau of isometric tetani. The controlled-velocity release method was used. Experiments were performed at about 2·25 μm sarcomere length and at 3–4 °C or at 19–21 °C.

2. Replacing H_2O with D_2O resulted in a rapid large reduction of the peak twitch tension and of the speed of development of twitch and tetanic tensions. The tetanic tension (P_0) was usually reduced, in certain fibres to as low as 5 % of the value in H_2O-Ringer solution.

3. The depression of twitch and tetanus characteristics was followed by a recovery, the duration of which varied greatly in different fibres. During the recovery period previous conditioning activity potentiated the tetanus characteristics.

4. After the end of the recovery period in D_2O-Ringer solution both the peak twitch tension and the speed of development of tetanic tension were still greatly depressed, whereas the value of P_0 was slightly greater than in H_2O-Ringer. The speed of rise of isometric tension after a quick release imposed at the tetanus plateau was reduced in D_2O-Ringer, usually to about 50 % of the value in H_2O-Ringer.

5. D_2O increased the development time of the $P-V$ relation and produced a conspicuous increase in the degree of its curvature. The value of V_0 (the velocity of shortening at zero load) was not significantly depressed by D_2O and it was the same independent both of the time after the beginning of stimulation and of the isometric tension at which the measurement was made. The $P-V$ relation attained its final characteristics before the isometric tension reached the plateau. During the recovery period in D_2O-Ringer, at the plateau of isometric tetani of different size, the relative force exerted at a given velocity of shortening was constant.

6. In D_2O-treated fibres, NO_3^- and caffeine (i) potentiated the peak twitch tension and the speed of development of tetanic tension without affecting significantly the speed of the redevelopment of tension after a quick release imposed at the tetanus plateau and (ii) reduced the development time of the $P-V$ relation, but did not affect either the degree of its curvature or the values of V_0 and P_0.

7. The results are discussed by assuming that the release of Ca^{2+} from the

sarcoplasmic reticulum is a rate-limiting process for the development of activation and in turn for the development of isometric tension. In terms of the cross-bridge model of Huxley (1957), the time or Ca^{2+}-dependent factor of activation appears to be the recruitment of actin sites for cross-bridge formation, whereas the value of the rate constants regulating the cross-bridge kinetics appears to be time and Ca^{2+}-independent.

INTRODUCTION

Previous work (Cecchi, Colomo & Lombardi, 1978; Cecchi, Colomo, Lombardi & Piazzesi, 1979) has shown that during the rising phase of an isometric tetanus the degree of activation (which was measured by the steady force exerted at any velocity of shortening lower than V_0) grows with time and attains its final characteristics before the tension reaches the plateau. In accordance with the predictions of the cross-bridge model of Huxley (1957), the slower time course of the rise of isometric tension with respect to that of the development of activation was mainly attributed to the moderate value of the rate constant controlling the attachment of cross-bridges.

The present paper deals with the factors controlling the rate of development of activation and, in turn, the speed of rise of isometric tension in intact frog muscle fibres. A first question which arises is whether the release of Ca^{2+} from the sarcoplasmic reticulum may represent a rate-limiting process for the development of both activation and isometric tension. Deuterium oxide was used because it affects the excitation–contraction coupling by depressing the Ca^{2+}-releasing mechanism of the sarcoplasmic reticulum (Goodall, 1958; Svensmark, 1961; Kaminer & Kimura, 1972; Eastwood, Grundfest, Brandt & Reuben, 1975; Sandow, Pagala & Sphicas, 1976; Yagi & Endo, 1976). A second question is whether the activation process implies only a recruitment of actin sites for cross-bridge formation or also an increase in the value of the rate constants regulating the kinetics of cross-bridges (Huxley, 1957; Julian, 1969, 1971; Podolsky & Teichholz, 1970; Julian & Sollins, 1973; Podolsky & Nolan, 1973; Lännergren, 1978; Cecchi *et al.* 1978).

A preliminary and partial report of the results has been already published (Cecchi, Colomo & Lombardi, 1979*a*).

METHODS

The techniques and methods of procedure are similar to those described in a previous paper (Cecchi *et al.* 1978) with the exception of the tension transducer.

Experiments were performed on directly stimulated single fibres isolated from the semitendinosus muscle of the frog (*Rana esculenta*). Stimuli of alternating polarity, 0·5 ms duration and 1·3 times the threshold strength were used. In order to minimize the amount of compliance in series with the sarcomeres special care was taken in mounting the fibres to make the lengths of the tendon attachments as short as possible. Data were collected first in normal Ringer solution and then in the test solution. In a few experiments this procedure was reversed. The force–velocity (P–V) relation was determined using the controlled-velocity release method. Releases were imposed at pre-set times during an isometric tetanus by means of the servo-system described previously (Cecchi, Colomo & Lombardi, 1976*b*). The tension was measured by means of a capacitance-gauge transducer similar to that described by Cecchi *et al.* (1979*b*). The resonant frequency of the transducers used ranged from 7 to about 11 kHz. In all the experiments the average sarcomere length of the resting muscle fibres was about 2·25 μm. The normal Ringer solution had the following composition: 115 mM-NaCl, 2·5 mM-KCl, 1·8 mM-CaCl, 3 mM-phosphate buffer at pH 7·1. The D_2O-Ringer solution was prepared by replacing about 99·9 % of the water in the normal Ringer (H_2O-Ringer) with D_2O (Uvasol, Merck). Nitrate solutions were prepared by isotonic substitution of $NaNO_3$ for

NaCl. Caffeine was added to the solutions. P_0 is the observed value for tetanic tension, whereas P_0^* is the intercept on the load axis of the calculated $P-V$ curve. P_t is the isometric tension developed during the tetanic contraction at the time when releases were imposed, l_0 is the fibre length at a sarcomere length of about 2·25 μm. V_0 (l_0/s) is the smallest velocity of release required to drop the isometric tension to zero or, conversely, the velocity of shortening under zero load.

RESULTS

(*A*) *Effects of D_2O on twitch and tetanus characteristics*

It was confirmed that replacing H_2O with D_2O greatly affects the characteristics of twitch and tetanic isometric contractions (Goodall, 1958; Kaminer, 1960; Svensmark, 1961; Sandow *et al.* 1976). The main effects of D_2O on characteristics of twitch and tetanic responses have been summarized in Figs. 1 and 2.

(1) *Early effects.* In accordance with the results of Yagi & Endo (1976) the action of D_2O was found to be fast. In all fibres examined, within the time required to change the bathing solution and to make the first records (15–20 s), the peak twitch tension and the rate of development of both twitch and tetanic tension were dramatically reduced. In twenty muscle fibres the peak twitch tension was reduced to 2–14 % of the value in H_2O-Ringer. Also the plateau tetanic tension was usually reduced, in certain fibres to as little as 5 % of the value in H_2O-Ringer, but in four fibres this effect appeared to be moderate or absent.

In fibres in which the early depressant action of D_2O was strong there was a decrease of 20–40 % in the optimal stimulus frequency, but the effect on the peak twitch tension and on the rate of development of isometric tension was so great that the number of stimuli required to raise the isometric tension to the plateau tetanic level was considerably greater in D_2O-Ringer than in H_2O-Ringer.

(2) *Recovery period.* In D_2O-Ringer, both at 2–4 °C and 19–20 °C, the depression of the various characteristics of twitch and tetanic responses was followed by a period of recovery. In all fibres examined the recovery of the peak twitch tension and of the rate of development of the tetanic tension was far from being complete. In addition, the recovery of the peak twitch tension and, in a tetanus, the recovery of the rate of development of tension were considerably slower than that of the plateau tension. The duration of the recovery of various contraction characteristics varied greatly in different muscle fibres. For instance, in the fibre of Fig. 1*A*, which was characterized by a rather slow recovery of tetanus characteristics, 16 and 60 min were required, respectively, for the recovery of the plateau tension and for the recovery of the rate of tension rise. On the other hand, in the fibre of Fig. 1*B*, 30 s after changing the bathing solution, the plateau tetanic tension was the same as in H_2O-Ringer and about 12 min was required for the recovery of the rate of the tension rise. It is possible, therefore, that in the fibres like that of Fig. 1*B*, in which the early depression of plateau tetanic tension appeared to be absent, recovery might have been practically complete within the time required to make the first records, but it is also possible that in these muscle fibres D_2O failed to depress the plateau tetanic tension.

A surprising effect of D_2O was that during the recovery period a tetanic volley, delivered to the muscle fibres with a short delay after a previous tetanus, produced a response in which both the amount and the rate of tension development appeared potentiated.

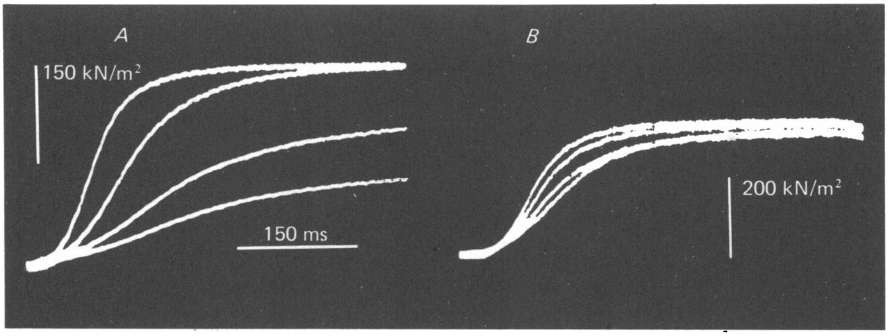

Fig. 1. Time course of the recovery of tetanus characteristics in D_2O-Ringer. Records refer to two muscle fibres at 20 °C (*A*) and at 19·5 °C (*B*). From bottom to top: *A*, 4, 8, 16 and 60 min after replacing H_2O-Ringer with D_2O-Ringer; *B*, 30 s, 5, 9 and 12 min after changing the bathing solution. Note that the recovery period of the plateau tension is shorter than that of the speed of the initial development of tension. Time calibration: 150 ms. Stimulation frequency: 66/s (*A*) and 110/s (*B*). Sarcomere length: 2·28 μm (*A*) and 2·25 μm (*B*). Major and minor fibre diameters: 80 and 62·5 μm (*A*): 72·5 and 45 μm (*B*).

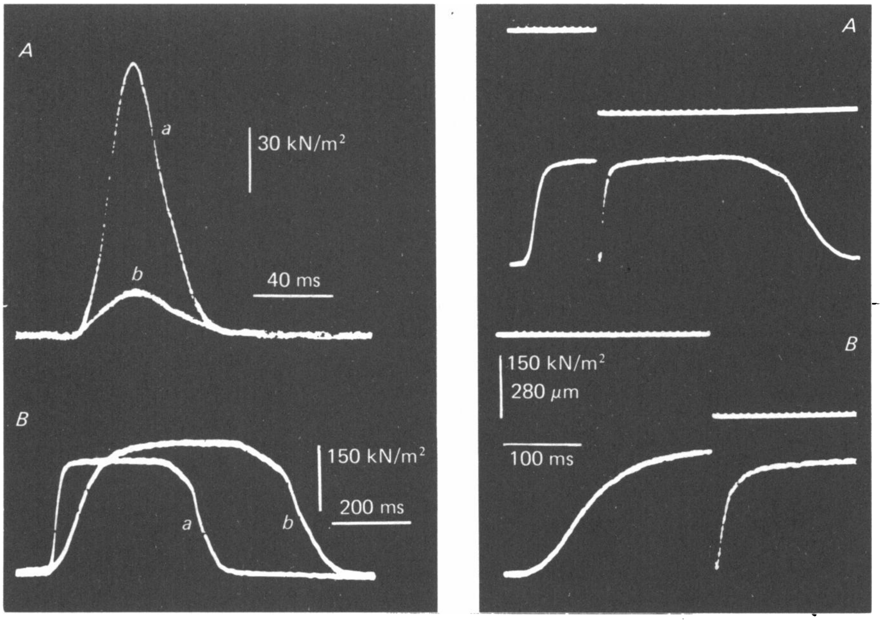

Fig. 2. Same muscle fibre as in Fig. 1 *B* (l_0: 9·37 mm; sarcomere length: 2·25 μm). Left-hand panel: effects of D_2O on characteristics of twitch (*A*) and tetanic (*B*) isometric contractions; *a*, control in H_2O-Ringer; *b*, 12 min after replacing H_2O-Ringer, when the recovery phase of twitch and tetanus characteristics was ended. Right-hand panel: effects of D_2O on the rate of the redevelopment of isometric tension after a quick release imposed at the tetanic plateau. *A*, control response in H_2O-Ringer; *B*, test response in D_2O-Ringer, 16 min after changing of the bathing solution. Upper traces (modulated by stimulus signals): fibre length; lower traces: tension. Note that in D_2O-Ringer the isometric tension fails to attain the same value as before the release.

During the recovery period in D_2O-Ringer there was a progressive increase in the optimal stimulus frequency, but it was noted that the number of stimuli required to raise the tetanic tension to its full level decreased as the rate of tension development increased.

(3) *Final effects.* At the end of the recovery period in D_2O-Ringer the plateau tetanic tension was slightly greater than in H_2O-Ringer. The amount of this potentiating effect of D_2O (in twenty fibres, about 8 %) was statistically significant, but it is much less than that described by Eastwood *et al.* (1975) for the mechanical response of skinned muscle fibres. The reason of this discrepancy is not clear. The above finding, moreover, disagrees also with the results of previous work showing that in the whole frog muscle D_2O significantly decreases the tetanic tension (Svensmark, 1961; Sandow *et al.* 1976), but this may be explained either by the relatively long exchange of D_2O with H_2O in the whole muscle (Svensmark, 1961) or by the very large variation observed in different fibres for the recovery time of plateau tetanic tension.

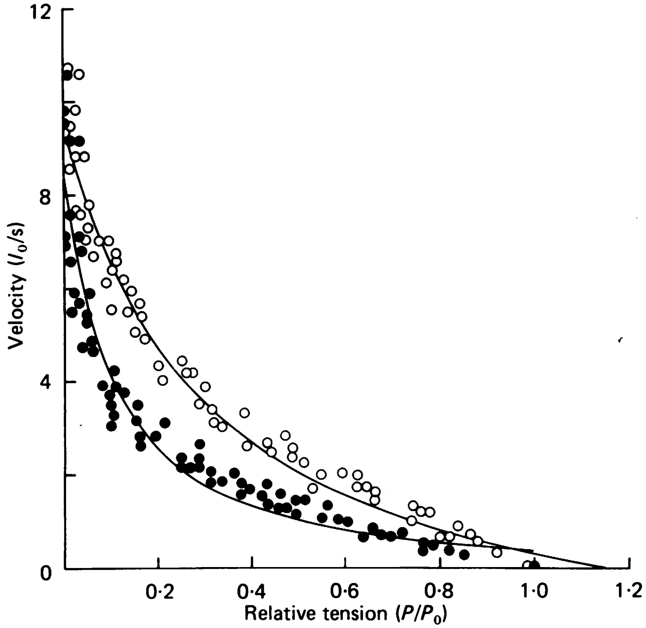

Fig. 3. P–V relations determined at the tetanus plateau in H_2O-Ringer (○) and in D_2O-Ringer (●). Data from eight fibres at room temperature. For each fibre the values of P were normalized for the corresponding value of P_0. The parameters of the P–V curves, drawn from Hill's hyperbolic equation, are listed in the following Table.

	V_0 (l_0/s)	P_0^*/P_0	a/P_0	b (l_0/s)
H_2O-Ringer	9·59	1·16	0·3	2·48
D_2O-Ringer	8·58	1·95	0·1	0·44

At the end of the recovery period the peak twitch tension was still considerably depressed (in different fibres to 7–26 % of the values in H_2O-Ringer) and the speed of development of tetanic tension was 4–5 times lower than in H_2O-Ringer, whereas the optimal stimulus frequency was again about the same as in H_2O-Ringer.

D_2O did not affect significantly the contraction twitch time and slightly reduced the rate of tetanus relaxation. D_2O also reduced by a factor of about two the rate of rise of isometric tension after a quick release imposed at tetanic plateau, but this depressant effect was much less severe than that observed during the rising phase of tetanus. Washing with H_2O-Ringer entirely restored the characteristics of twitch and tetanus contractions within several minutes.

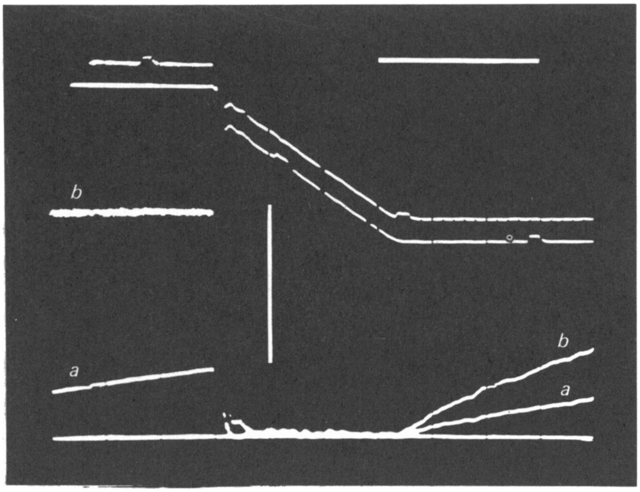

Fig. 4. To show that in D_2O-Ringer as well as in H_2O-Ringer (Cecchi *et al.* 1978) V_0 is independent both of time after the beginning of stimulation and of the tension developed during the rise of an isometric tetanus. The upper traces, modulated by stimulus signals, refer to releases imposed 40 and 350 ms after the start of stimulation when the isometric tension had risen, respectively, to 0·27 P_0 (*a*) and to P_0 (*b*). The lower horizontal trace is the resting tension. The velocity of shortening for both releases was the same, 4·82 l_0/s. Note in the records the absence of delay between the end of release and the start of the redevelopment of isometric tension. Vertical calibration: 285 kN/m^2 or 410 μm; horizontal calibration: 7·5 ms. Stimulation frequency: 105/s in H_2O-Ringer and 82/s in D_2O-Ringer. Sarcomere length: 2·25 μm. l_0: 12·1 mm. Major and minor fibre diameters: 112 and 71 μm.

(*B*) *Effects of D_2O on the P–V relation at the tetanus plateau*

Fig. 3 summarizes the results from eight experiments performed at room temperature. P–V data in D_2O-Ringer were obtained after waiting for the end of the recovery of tetanus and twitch characteristics. Loads were expressed in relative units so that the small potentiation effect of D_2O on tetanic tension was ignored. It can be seen that the value of V_0 was not significantly depressed by D_2O and that, in spite of the failure of Hill's hyperbola (1938) to give an acceptable fit, the main effect of D_2O is a substantial increase in the degree of curvature of the P–V relation or, in other words, a decrease in the value of Hill's constant a/P_0^*.

(*C*) *Effects of D_2O on the rate of development of the P–V relation*

In these experiments (Figs. 4–6) the P–V relation was determined at pre-set times after the beginning of the tetanus volley either during the rise of tension or at the plateau. Data points in D_2O-Ringer were obtained after waiting for the end of the recovery of tetanus and twitch characteristics. In accordance with what was observed in H_2O-Ringer (Cecchi *et al.* 1978, 1979), in D_2O-Ringer during the tetanus rise the value of V_0 was independent of time after the start of stimulation (Fig. 4), conversely, the force P exerted during shortening at any velocity lower than V_0 increased with time and attained its full value before the isometric tension. However, both the rate of development of P and its final value were much lower in D_2O-Ringer than in H_2O-Ringer.

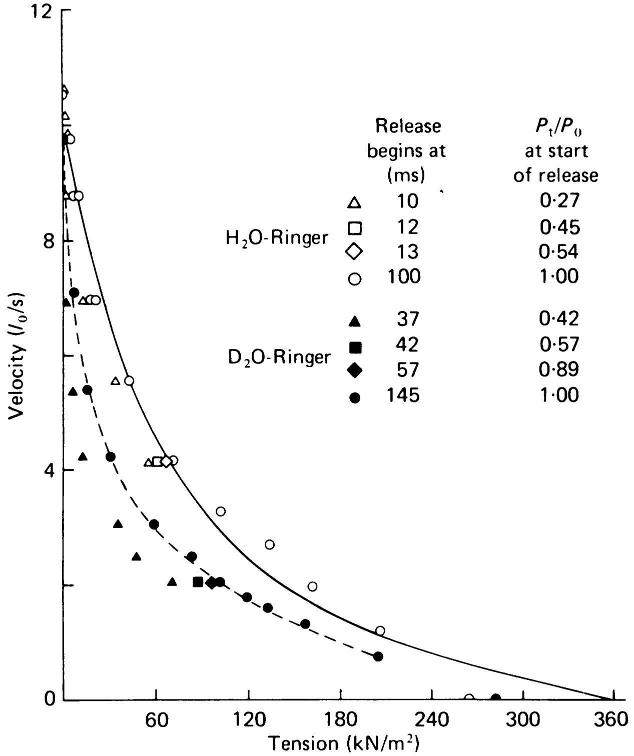

Fig. 5. P–V relations at various times during isometric tetanic contractions in H_2O-Ringer (open symbols) and in D_2O-Ringer (filled symbols). Circles refer to releases imposed at the tetanus plateau; triangles, squares and rhombi refer to releases imposed during the tension rise. The continuous curve was fitted to data points obtained at the tetanic plateau in H_2O-Ringer (○) by means of Hill's hyperbolic equation. The interrupted curve lying on data points obtained at the tetanus plateau in D_2O-Ringer (●) was drawn by eye because of the failure of Hill's hyperbola to give an acceptable fitting. Stimulation frequency: 125/s both in H_2O-Ringer and in D_2O-Ringer. Sarcomere length: 2·25 μm. l_0: 14·6 mm. Major and minor fibre diameters: 82·5 and 77·5 μm.

Fig. 5 illustrates the results obtained from a fibre in which the rate of development both of the *P–V* relation and of the tetanic tension in H_2O-Ringer were rather fast. In this fibre at 20·5 °C the time required by the *P–V* relation to attain its plateau characteristics in H_2O-Ringer was about 13 ms, and at this time the isometric tension had risen to 0·54 P_0. Bathing of the fibre with D_2O-Ringer increased the development time of the *P–V* relation to about 57 ms and by this time the isometric tension had risen to 0·89 P_0, a considerably greater value than in H_2O-Ringer. Fig. 6 shows the

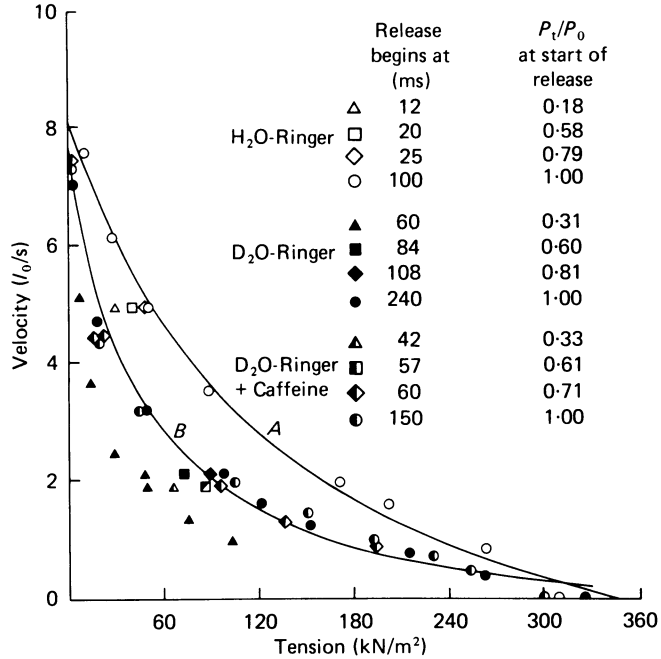

Fig. 6. Effects of caffeine (2 mM) on the rate of development of the *P–V* relation in a D_2O-treated fibre at 19 °C. Circles refer to releases imposed at the tetanus plateau either in H_2O-Ringer (curve *A*, ○) or in D_2O-Ringer both before (curve *B*, ●) and after addition of caffeine (◐). Both curves were fitted to data points by Hill's hyperbolic equation. The other symbols (triangles, squares and rhombi) refer to releases imposed at shorter times or lower isometric tensions during the tetanus rise in H_2O-Ringer (open symbols), in D_2O-Ringer (filled symbols) and in D_2O + caffeine – Ringer (half-filled symbols). Stimulation frequency: 105/s. Sarcomere length: 2·25 μm. l_0: 11 mm. Major and minor fibre diameters: 70 and 55 μm.

results obtained from another fibre in which the rate of development of both the *P–V* relation and the tetanic tension in H_2O-Ringer was slower. In this fibre at 19 °C the time required by the *P–V* relation to attain its plateau characteristics was longer, about 25 ms, and at this time the isometric tension had risen to 0·79 P_0. Data in Fig. 6 bear out those in Fig. 5 showing that D_2O increased the development time of the *P–V* relation from about 25 to about 108 ms. At this time the isometric tension was 0·81 P_0, a value which is comparable to that observed in H_2O-Ringer. In general, depending on the rate of development of the *P–V* relation in H_2O-Ringer, D_2O either increased or did not alter the tension value at which during the tetanus rise the *P–V* relation attained its plateau characteristics.

(*D*) *The P–V relation at the tetanic plateau during the recovery of contraction characteristics in* D_2O-*Ringer*

Previous work (Cecchi *et al.* 1978, 1979) has shown that during the rise of the tension in an isometric tetanus the P_0 value increases with time after the beginning of the stimulus volley, whereas the values of the other P–V characteristics, V_0 and a/P_0, appear to remain constant. On the other hand, at a low initial isometric tension, because of possible errors due to the large extrapolation required to intercept the load axis, the P_0 value and, therefore, also the a/P_0 value may be unreliable. The observation that in certain fibres D_2O produced an early drop of the tetanic tension followed by a slow recovery gave the opportunity to verify whether, under steady-state conditions of activation at the plateau of tetanic contractions, the value of a/P_0 depends on the level of isometric tension at which the measurements are made. A disadvantage of this procedure is that, because of the recovery of tetanus charac-

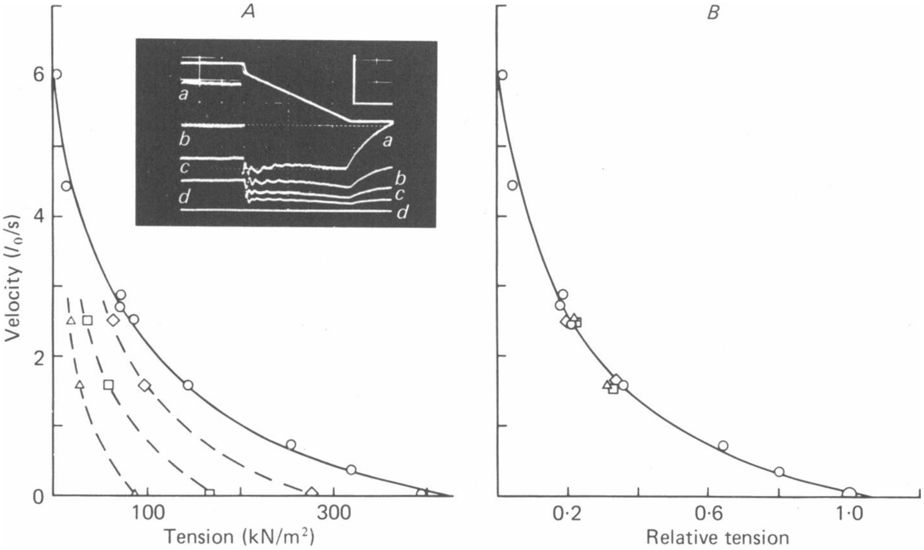

Fig. 7. Absolute (*A*) and relative (*B*) P–V relations determined in a fibre at 19·5 °C at the plateau of isometric tetani of different sizes in D_2O-Ringer. Circles refer to data points obtained at the end of two successive recovery periods in D_2O-Ringer, when the isometric tetanic tension had attained a constant level (P_0). The other symbols refer to data points obtained during both recovery periods, when the tetanic tension was 0·23 P_0 (triangles), 0·42 P_0 (squares) and 0·7 P_0 (rhombi). The velocity of shortening was 1·62 l_0/s during the first recovery period and 2·25 l_0/s during the second one. For each set of data points the scaling factor for expressing loads in relative units was the actual value of plateau tension. The continuous curves were fitted to data points by Hill's hyperbolic equation. The interrupted curves were fitted to data points by eye. In the inset the top trace measures the fibre length; the records *a*, *b*, *c* and *d* are responses to releases at 1·62 l_0/s imposed at the tetanic plateau during the first recovery period in D_2O-Ringer. The lower horizontal trace is the resting tension. Vertical calibration: 150 kN/m² or 470 μm; horizontal calibration: 9 ms. Stimulation frequency: 100/s. Sarcomere length: 2·27 μm. l_0: 12·4 mm. Major and minor fibre diameters: 87·5 and 62·5 μm.

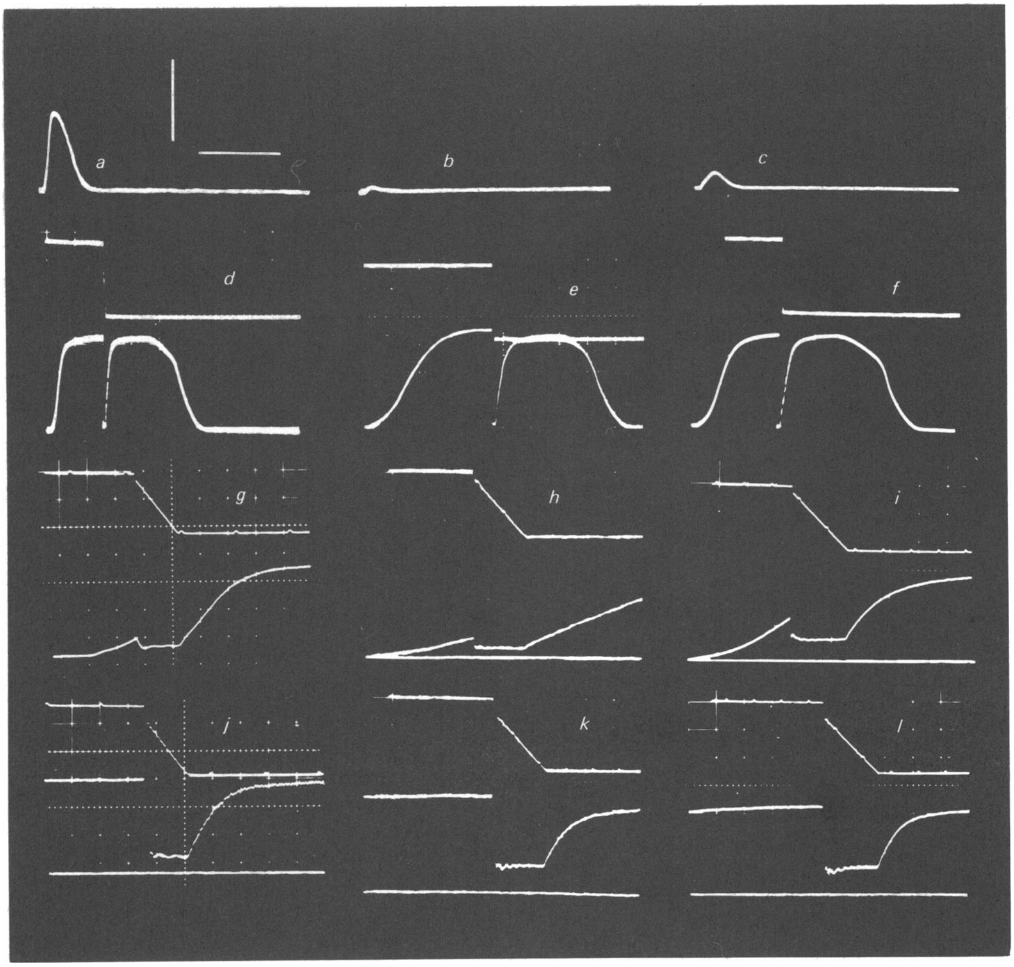

Fig. 8. Sample records from the same experiment illustrated in Fig. 6. First column, in H_2O-Ringer; second column, in D_2O-Ringer; third column, in D_2O-caffeine-Ringer. Records in *a*, *b* and *c* refer to twitch responses. Records in *d*, *e* and *f*, refer to tetanic responses (bottom traces) at the plateau of which quick releases were imposed (top traces). Records in *g*–*l* refer to responses (bottom traces) to controlled-velocity releases (top traces) imposed at pre-set times during an isometric tetanus either at a low initial tension (*g*, *h*, *i*) or at the plateau (*j*, *k*, *l*). The velocity of shortening was 4·94 l_0/s in H_2O-Ringer, 2·14 l_0/s in D_2O-Ringer and 1·97 l_0/s in D_2O-caffeine-Ringer. The lower horizontal traces are the resting tensions. Vertical calibration: 150 kN/m^2 (*a*–*c*) and 300 kN/m^2 or 620 μm (*d*–*l*). Horizontal calibration: 150 ms (*a*–*f*); 15 ms (*g* and *j*); 30 ms (*h*, *i*, *k* and *l*).

teristics, only one P–V point could be determined at each tension level. Fig. 7 shows the results obtained from one fibre at room temperature. It can be seen that the greater the plateau tetanic tension at which controlled-velocity releases were imposed, the greater also the force exerted during shortening, but the relative force exerted at a given velocity of shortening, and therefore the value of a/P_0, remained constant.

(*E*) *Effects of NO_3^- and caffeine on the mechanical performance of D_2O-treated muscle fibres*

NO_3^- (50 mM) and caffeine (1–2 mM) were used because in normal muscle they affect excitation–contraction coupling by increasing the amount of Ca^{2+} available for activation at the level of myofilaments. Bathing a fibre with NO_3^- or caffeine solution was commenced after waiting for the end of the recovery of twitch and tetanus characteristics in D_2O-Ringer. All the results reported here refer to experiments made at room temperature and to data obtained after waiting for the potentiating action of NO_3^- and caffeine to become maximal.

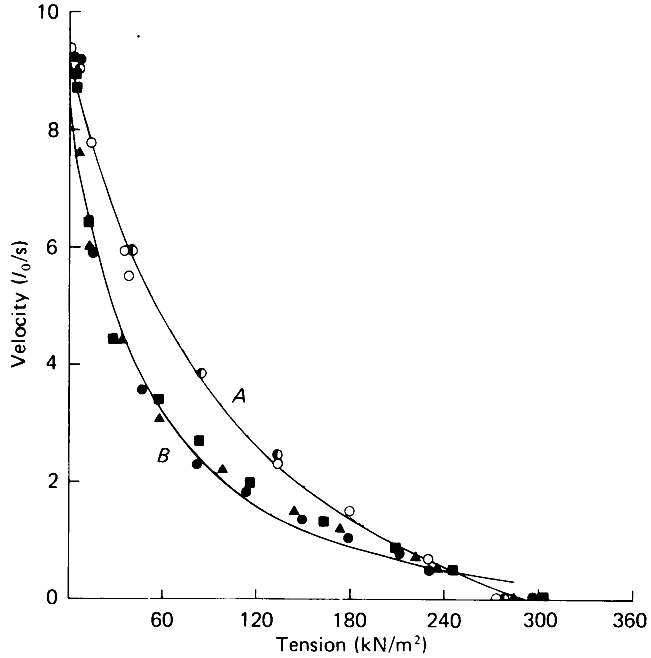

Fig. 9. Absence of significant effects of NO_3^- (50 mM) and of caffeine (1 mM) on the characteristics of the plateau *P–V* relation in D_2O-treated fibres. Data from a fibre at 19 °C. ○, ◑ (curve *A*) refer to data points obtained in H_2O-Ringer, respectively before and after treatment of the fibre with D_2O. Filled symbols refer to data obtained in D_2O-Ringer either before (●) or after addition of NO_3^- ions (curve *B*, ▲) or caffeine (■). Curves were drawn using Hill's hyperbolic equation. In each bathing solution data points were determined after waiting for the contraction characteristics to settle to a steady level. Stimulation frequency: 105/s in H_2O-Ringer and 93/s in D_2O-Ringer. Sarcomere length: 2·25 μm. l_0: 11·2 mm. Major and minor fibre diameters: 100 and 75 μm.

The effects of caffeine on the mechanical performance of D_2O-treated fibres are shown in Figs. 6 and 8, but comparable results were also obtained with NO_3^- ions. Fig. 9 concerns another experiment in which it was possible to study the effects of NO_3^- and caffeine on the same D_2O-treated fibre. In general, in D_2O-treated fibres NO_3^- and caffeine produced a moderate recovery of peak twitch tension and increased by a factor of about two the rate of development of tetanic tension, but did not affect significantly either tetanic tension or the rate of the redevelopment of tension after a quick release imposed at the tetanic plateau. Also the value of V_0 and the degree of curvature of the P–V relation were not affected by NO_3^- and caffeine. In this respect the behaviour of D_2O-treated fibres is similar to that of normal fibres (Cecchi *et al.* 1978; G. Cecchi, F. Colomo & V. Lombardi, unpublished results). NO_3^- and caffeine only partially reversed the depressant effects of D_2O on the rate of development of the P–V relation. For instance, in the fibre of Fig. 6 (in which the full development time of the P–V relation in H_2O-Ringer required about 25 ms) addition of caffeine to the D_2O-Ringer solution reduced the development time of the P–V relation from about 108 to about 60 ms and at this time the isometric tension had risen to 0·71 P_0, a slightly lower value than that observed either in H_2O-Ringer (0·79 P_0) or in D_2O-Ringer before addition of caffeine (0·81 P_0). In general, in D_2O-treated fibres NO_3^- and caffeine considerably increased the rate of development of the P–V relation, without affecting significantly the tension value at which the P–V relation attained its final characteristics. Finally, before and after treatment with D_2O and NO_3^- or caffeine, the time of development of the P–V relation and the amplitude of the peak twitch tension exhibited a highly significant inverse correlation.

DISCUSSION

In accordance with previous work (Kaminer & Kimura, 1972; Eastwood *et al.* 1975; Sandow *et al.* 1976; Yagi & Endo, 1976) most of the results of the present paper may be explained by assuming that D_2O reduces the amount of Ca^{2+} released from sarcoplasmic reticulum by individual stimuli and in turn the rate of development of activation. D_2O in fact increased the number of stimuli required for complete development of the P–V relation and of the tetanic tension; moreover, during the recovery of contraction characteristics in D_2O-Ringer previous conditioning activity potentiated both the amount and the speed of development of tetanic tension; finally, the depressant effects of D_2O on the rate of development of the P–V relation, on the speed of rise of tension in twitch and tetanic contractions and on the amplitude of twitch response were partially reversed by NO_3^- and by caffeine. Since similar potentiating effects of NO_3^- and caffeine are present also in H_2O-Ringer (Sandow & Preiser, 1964; Cecchi, Colomo & Lombardi, 1976*a*, 1978; G. Cecchi, F. Colomo & V. Lombardi, unpublished results), it is likely that in normal fibres, as well as in D_2O-treated fibres, the release mechanism of Ca^{2+} from the sarcoplasmic reticulum represents a rate-limiting process for the development of activation and in turn for the development of isometric tension. An obvious consequence is that at least a part of the large variation observed in H_2O-Ringer, either in the times required by activation and by tetanic tension to attain their final levels, or in the amplitude of the peak twitch tension (Cecchi, Colomo & Lombardi, 1976*c*; Cecchi *et al.* 1978), depends on the variation of the rate of mobilization of activating Ca^{2+}. The finding, in each individual fibre before and after treatment with D_2O and NO_3^- or caffeine, of a highly significant inverse correlation between the time of development of the P–V relation and the peak twitch tension agrees with this view.

The incomplete recovery by treatment with NO_3^- or caffeine of the rate of development of the P–V relation in D_2O-treated fibres might be due to failure of these potentiator agents to restore entirely the mechanism of Ca^{2+} release. On the other hand, it is also possible that D_2O, besides decreasing the rate of release of Ca^{2+}, depresses *per se* the kinetics of the successive steps of the activation process. For instance, it could be that the rate constant regulating the binding of Ca^{2+} to the contractile proteins is directly depressed by D_2O.

The cause of the recovery of contraction characteristics in D_2O-Ringer is not clear. The contribution of a transitory rise in both the internal osmotic pressure and the ionic strength of muscle fibres, because the diffusion coefficient is higher for H_2O than for D_2O (Pinson, 1952), does not seem to be significant. Replacing H_2O-Ringer with D_2O-Ringer did not produce appreciable reduction in the fibre diameter. The correlation coefficient between fibre diameter and recovery time was insignificant and the large variation in the duration of the recovery period can not be explained by differences in the degree of cleanness of individual fibres.

Mechanisms of activation based on the cross-bridge model of Huxley (1957) and involving an increase either in the number of actin sites available for cross-bridge formation or in the values of the rate constants for the making and breaking of cross-bridges (Julian, 1969; Julian & Sollins, 1973; Podolsky & Nolan, 1973; Julian & Moss, 1976) have already been used to explain the time or Ca^{2+} or tension dependence of the characteristics of the P–V relation both in 'skinned' or 'glycerinated' fibres (Podolsky & Teichholz, 1970; Julian, 1971) and in intact fibres (Julian & Sollins, 1973; Lännergren, 1978; Cecchi *et al.* 1978, 1979). The result that, during the recovery of contraction characteristics in D_2O-Ringer, the relative force at a given velocity of shortening was the same, independent of the tetanic tension at which releases were imposed, shows that, under steady-state conditions at least, P_0^* is the sole P–V parameter which changes with the level of activation. Thus, in accordance with the view of Podolsky and his colleagues, also during the development of the contractile process in an intact fibre, the sole effect of Ca^{2+} release by action potentials should be an increase in P_0^* or, in terms of the cross-bridge model, a recruitment of actin sites for cross-bridge formation. The finding that during the initial rise of tetanic tension the value of V_0 remains constant, whereas the extrapolated value of P_0^* appears to grow with time after the beginning of stimulation (Cecchi *et al.* 1978) agrees with this view.

D_2O also produced a decrease in the value of a/P_0 without affecting significantly the value of V_0. Since the P_0 value was scarcely affected by D_2O, in terms of the cross-bridge model of Huxley (1957) the above finding implies a decrease in the values of the rate constants, f_1 and g_1, for attachment and detachment of cross-bridges. In this way it can be also explained how D_2O reduces the speed of the redevelopment of the isometric tension after a quick release imposed at the tetanus plateau. The much more severe depression by D_2O of the peak twitch tension and of the speed of the initial development of tetanic tension must be attributed to the concomitant slowing down of the recruitment of actin sites for cross-bridge formation, because of the depression of the Ca^{2+} release. As to the nature of the above effects of D_2O, in terms of the cross-bridge model of Huxley (1957), the observation that in D_2O-treated fibres NO_3^- and caffeine did not restore the value of a/P_0 implies that f_1 and g_1 are independent of the actual concentration of activating Ca^{2+} ions at the level of myofilaments and, therefore, that D_2O affects directly, *per se*, f_1 and g_1. This conclusion agrees with the view (see above) that P_0^* represents the only activation factor which appears to be time- and Ca^{2+}-dependent.

Finally, the finding that in fibres bathed with D_2O-Ringer the tension value at which the P–V relation attained its steady-state level was never found to be significantly lower than in normal fibres is not surprising. According to Huxley's model a decrease in f_1 and g_1, like that produced by D_2O, could have reduced this tension value only if the actual rate of development of activation had not been decreased.

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