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CALCIUM-ACTIVATED FORCE RESPONSES IN FAST- AND SLOW-TWITCH SKINNED MUSCLE FIBRES OF THE RAT AT DIFFERENT TEMPERATURES

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SUMMARY

1. Force responses from mechanically skinned fibres of rat skeletal muscles (extensor digitorum longus and soleus) were measured at different temperatures in the range 3–35 °C following sudden changes in Ca^{2+} concentration in the preparations.

2. At all temperatures there were characteristic differences between the slow- and fast-twitch muscle fibres with respect to the relative steady-state force–$[Ca^{2+}]$ relation: such as a lower $[Ca^{2+}]$ threshold for activation and a less steep force–pCa curve in slow-twitch muscle fibres.

3. At 3–5 °C the force changes in both types of muscle fibre lagged considerably behind the estimated changes in $[Ca^{2+}]$ within the preparations and this enabled us to perform a comparative analysis of the Ca^{2+} kinetics in the process of force development in both muscle fibre types. This analysis suggests that two and six Ca^{2+} ions are involved in the regulatory unit for contraction of slow- and fast-twitch muscle fibres respectively.

4. The rate of relaxation following a sudden decrease in $[Ca^{2+}]$ was much lower in the slow-twitch than in the fast-twitch muscle at 5 °C, suggesting that properties of the contractile apparatus could play an essential role in determining the rate of relaxation *in vivo*.

5. There was substantial variation in Ca^{2+} sensitivity between muscle fibres of the same type from different animals at each temperature. However the steepness of the force–$[Ca^{2+}]$ relation was essentially the same for all fibres of the same type.

6. A change in temperature from 5 to 25 °C had a statistically significant effect on the sensitivity of the fast-twitch muscle fibres, rendering them less sensitive to Ca^{2+} by a factor of 2. However a further increase in temperature from 25 to 35 °C did not have any statistically significant effect on the force–$[Ca^{2+}]$ relation in fast-twitch muscle fibres.

7. The effect of temperature on the Ca^{2+} sensitivity of slow-twitch muscle fibres was not statistically significant, mainly because of the large variation in sensitivity amongst these preparations at room temperature.

8. Two types of oscillatory processes not associated with intracellular membranes were observed in the force response of all slow-twitch muscle fibres when submaximally activated ($< 60\%$ maximum force) at 25 and 35 °C, but never at 3–5 °C. The frequency of oscillations increased with temperature.

9. Maximum Ca^{2+}-activated force in both muscle fibre types was greatly dependent upon temperature over the range 0–25 °C, but increased only slightly above 25 °C.

10. Experiments on the rigor state suggest that the number of possible actomyosin interacting sites diminishes considerably as temperature is decreased below 25 °C.

INTRODUCTION

The mechanical performance of an intact mammalian skeletal muscle fibre depends upon the fibre type (slow- or fast-twitch; Hess, 1970; Close, 1972) and temperature (Cullingham, Lind & Morton, 1960; Truong, Wall & Walker, 1964; Isaacson, Hinkes & Taylor, 1970; Close, 1972; Hill, 1972). Preliminary observations on mechanically skinned rat muscle fibres at 5 °C and 0·1 mM-Mg^{2+} (Stephenson & Forrest, 1980) have shown that some of the physiological differences between the two fibre types could be at least partly accounted for by differences in certain properties of the calcium-activated force responses of the respective muscle fibres. In the present work we have extended these studies to a wider range of temperatures (3–35 °C) and to a higher, more physiological level of Mg^{2+} (1 mM). The results confirm that the process of Ca^{2+} activation of force is different in the slow- and fast-twitch muscle fibres and indicate that temperature can affect this process differentially in these mammalian muscle fibres. Parts of this work have already been communicated elsewhere (Stephenson & Forrest, 1979; Stephenson & Williams, 1980*a*; Williams & Stephenson, 1980).

METHODS

Isolation and preparation of fibres. Adult rats (*Rattus norvegicus*) of either sex were killed by decapitation. The soleus and extensor digitorum longus muscles (e.d.l.) from both hind limbs were rapidly excised, thoroughly blotted and placed in cold (5 °C) paraffin oil (Ajax Chemicals). The muscles were kept in paraffin oil at 5 °C until used for preparing single skinned fibres. The dissection of single muscle fibres and the subsequent mechanical skinning were performed with the muscle under paraffin oil and on a bed of Sylgard 184 (Dow Chemicals) using the standard techniques employed in this laboratory (Ashley & Moisescu, 1977; Moisescu & Thieleczek, 1978). The fibres were always dissected from the medial region of the respective muscle.

Measuring and recording apparatus. The mechanical arrangements for attaching the preparation to the force transducer, recording force, and activation were similar to those previously described by Moisescu & Thieleczek (1978). The sensitivity of the force measuring system, which included an Aksjeselskapet AE 875 piezo-resistive force transducer, was normally 5V N^{-1} but could be varied. The natural frequency of the system was higher than 180 s^{-1}, depending upon the particular stainless-steel hook attached (with Shellac) to the original peg of the transducer. The total drift of the force recording system after the warming-up period (30 min) was below 3 nN s^{-1} and the extraneous compliance was below 10 mm/N. The maximal forces recorded with this system were in the range 0·1–1 mN.

For routine determinations of the sarcomere length we used the diffraction pattern produced by a He-Ne laser (Spectra-Physics 136–04) beam crossing the preparation. The solutions in which the sarcomere length was estimated from the laser diffraction pattern were contained in modified polystyrene spectrophotometric vials. The average sarcomere length d was calculated from the position of the first-order diffraction maxima using the approximate expression (1) which included a correction factor for the passage of the diffracted laser beam through the solution around the preparation and through the wall of the spectrophotometric vial before propagation though the air:

$$d = \lambda[1 + 4(d_{air} + n_w d_w + n_v \cdot d_v)^2 \cdot d_{11}{}^{-2}]^{0.5} \quad (1)$$

where λ is the wave-length (0·6328 μm); d_w (3–5 mm), d_v (1·5 mm), d_{air} (250–400 mm) the distances

travelled by the zero-order diffracted beam through the solution, polystyrene wall of the vial, and air respectively before reaching the screen; n_w and n_v are the refractive indices of water and polystyrene respectively and d_{11} the distance between the centres of the first-order diffraction maxima measured on the screen.

The sarcomere lengths derived by the diffraction technique agreed with independent miscroscopic measurements made on the same preparations.

Solutions. The bathing solutions were prepared using the same procedures previously described by Ashley & Moisescu (1977) and Moisescu & Thieleczek (1978) and the composition of the solutions is indicated in Table 1.

TABLE 1. Composition of main solutions. All solutions except NA and NB contained (mM): K^+ (117); Na^+ (36); Mg^{2+} (1); caffeine (10); NaN_3 (1). The pH of solutions was adjusted to 7·10±0·01 at the temperatures indicated such that the variation in total $[K^+]$ was not more than ±1 mM between solutions

Solution	ATP_{total} (mM)	Mg_{total} (mM)	CP (mM)	CK (u./ml)	HDTA (mM)	EGTA (mM)	CaEGTA (mM)	TES (mM)	Temp. (°C)
A	8·0	8·0	10	15	—	—	50	140	2
B	8·0	9·2	10	15	—	50	—	140	2
H	8·0	8·4	10	15	50	—	—	140	2
A′	8·0	8·12	10	15	—	—	50	60	22
B′	8·0	10·30	10	15	—	50	—	60	22
H′	8·0	8·50	10	15	50	—	—	60	22
A″	8·0	8·40	10	15	—	—	50	40	35
B″	8·0	11·00	10	15	—	50	—	40	35
H″	8·0	8·70	10	15	50	—	—	40	35
R	—	2·4	—	—	18	50	—	140	2
R′	—	3·35	—	—	18	50	—	60	22
NA*	6·0	6·3	10	15	—	—	—	100	5
NB*	6·0	6·12	10	15	—	0·1	—	100	5

* NA contained 30 mM-nitrilotriacetic acid. The main anion in NB was Cl^-. Both solutions also contained: 1 mM-Mg^{2+}; 33 mM-Na^+; 115 mM-K^+; at pH 7·10.

Abbreviations: HDTA – hexamethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid: EGTA – ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; TES – 2-(2-hydroxy-1,1-bis(hydroxymethyl) ethyl) aminoethane sulphonic acid; ATP – adenosine-5-triphosphate; CP – creatine phosphate; CK – creatine phosphokinase; $pCa = -\log_{10}[Ca^{2+}]$.

All substances were of analytical grade. We chose as standard conditions for these experiments the following cationic concentrations (mM); Mg^{2+}, 1; K^+, 117; Na^+, 36; pH 7·10. These values are very close to those considered to exist in intact vertebrate skeletal muscle (see Moisescu & Thieleczek, 1978). The solutions also contained caffeine (10 mM) and azide (NaN_3, 1 mM) in order to reduce the Ca^{2+} movements associated with the sarcoplasmic reticulum and mitochondria (Jewell & Rüegg, 1966). The apparent affinity constants used for preparing the solutions were either previously determined in our laboratory (Moisescu & Thieleczek, 1978, 1979) or were specifically measured for this set of experiments using the potentiometric methods described by Moisescu & Thieleczek (1979), the latter values being indicated in Table 2.

In order to increase accuracy in estimating the relative and absolute Ca^{2+} concentrations in the solutions, we have not only titrated with $CaCl_2$ the amount of EGTA in excess of Ca in every solution or combination of solutions used (Moisescu & Thieleczek, 1978), but have also estimated the *total* concentration of EGTA and HDTA in our solutions by employing $CdSO_4$ for titrations. Two examples of such titrations with $CdSO_4$, one for EGTA and the other for HDTA, are illustrated in Fig. 1 *A* and *B* respectively. When titrating HDTA, diluted to 1–3 mM, the pH end-point for titration should be in the pH range 7–8, so that the apparent affinity constant of Cd^{2+} to HDTA, and the number of protons displaced following the binding of Cd^{2+} to HDTA are both high enough to enable accurate estimations. However, when titrating total concentrations of EGTA, diluted to 1–10 mM, in the presence of different amounts of Ca, then the pH end-point should be between 4

and 4·5. A pH end-point outside this range would result in poorer resolution due to either a substantially higher apparent affinity constant of Ca^{2+} to EGTA, or a substantially lower apparent affinity constant of Cd^{2+} to EGTA. With these titrations we were also able to balance the various solutions more accurately, particularly when replacing EGTA with HDTA, since both of these substances are hygroscopic to some degree. Furthermore, we could determine and limit the extent of evaporation (or condensation) in our solutions during an experiment to within ±2 %All solutions were freshly prepared for each experiment and were normally used within 12 h (Moisescu &

TABLE 2. Measured values for the apparent affinity constants (K'_Lapp) of ATP, HDTA and EGTA for Ca^{2+} and Mg^{2+} at 35 °C using the potentiometric methods of Moisescu & Thieleczek (1979).

			Experimental conditions		
Ligand	Cation	K'_Lapp (M^{-1})	K^+ (mM)	Na^+ (mM)	pH
EGTA	Ca^{2+}	$(5·05 \pm 0·5) \times 10^6$	117	36	7·10*
	Mg^{2+}	55 ± 5	117	36	7·10
ATP	Ca^{2+}	$(3·5 \pm 0·3) \times 10^3$	117	36	7·10
	Mg^{2+}	$1·0 \pm 0·1 \times 10^4$	117	36	7·10
HDTA	Mg^{2+}	6 ± 2	117	36	7·10

* Measured at pH 5·5–5·8 and extrapolated to pH 7·10. For other temperatures we have used the apparent affinity constants measured with similar methods (Ashley & Moisescu, 1977; Moisescu & Thieleczek, 1978) adjusted for our experimental conditions.

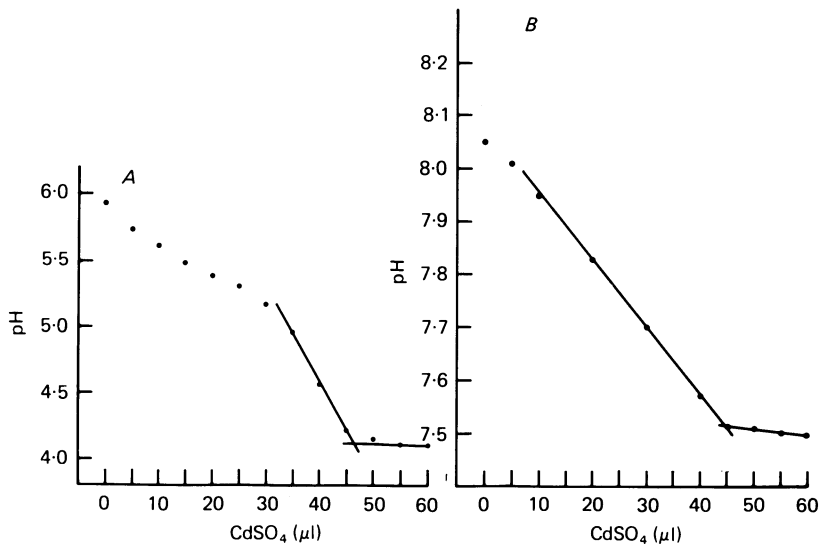

Fig. 1. Typical titration curves with $CdSO_4$ (0·3 M) of *A*, EGTA total (*ca.* 80 %present as CaEGTA) and *B*, HDTA, in experimental solutions of type A/B and H respectively, diluted 1:9 with a solution containing 200 mM-KCl, 10 mM-TES, 20 mM-$MgCl_2$. The intersections of the lines in *A* and *B* correspond to the end-points of titrations.

Thieleczek, 1978). Heavily used solutions were replaced several times during the course of each experiment to avoid significant changes in their composition due to contamination. The pH was routinely checked in each solution before, during and after each experiment, using an Orion 701A digitical pH meter. If the difference in pH between solutions increased beyond ±0·02 pH units the respective solutions were replaced.

The temperature was maintained constant during each experiment using a water/glycerol mixture circulating around the solutions after passing through a refrigerating unit and/or a

thermostat bath as previously described (Moisescu & Thieleczek, 1978). The difference in temperature between solutions was regularly checked and did not exceed 0·5 °C between any two solutions during an experiment at a given temperature. However, the temperature might have varied by ±1·5 °C between different experiments at 'low' (3–5 °C) and 'room' (22–25 °C) temperatures. Experiments were also conducted in a thermostatically controlled cold room (5 °C) to avoid any possible changes in the temperature of the preparation, particularly during the movement of the preparation through the air–solution interface when changing solutions.

Protocol for data collection

After being attached to the force transducer, the skinned muscle fibres were aligned and then allowed to equilibrate for at least 10 min in a relaxing solution containing free EGTA (50 mM). A solution with a Ca^{2+} concentration below 2×10^{-8} M, in which no active force could be detected, is referred to as relaxing solution. Then, the sarcomere length was usually adjusted to 2·7–2·8 μm which corresponds to the optimum initial length for contraction *in vivo* in these muscles (Close, 1972), and the dimensions of the preparation (diameter and length) were determined.

The average length of the sarcomeres was measured again after 5–6 contraction–relaxation cycles and the fibre was used for further experiments only if it appeared homogeneous along its entire length and if the sarcomere length was not substantially different from the initial adjusted value. The preparations were also discarded if the maximum force dropped by more than *ca.* 10% between two consecutive control maximum contractions. Despite the relatively small deterioration in force for each set of responses at a given sarcomere length, we always corrected the graph data on relative force responses by normalizing to interpolated control contractions using a method similar to that of Julian (1971).

RESULTS

As has been noticed with other invertebrate and vertebrate muscle preparations (Stephenson & Williams, 1980*b*; Moisescu & Thieleczek, 1979), there is an intrinsic variation in the Ca^{2+} sensitivity of rat myofibrillar bundles from the same fibre type of different animals, even when employing identical experimental conditions (composition of solutions, temperature, sarcomere length). In addition, we occasionally encountered soleus and e.d.l. preparations which had Ca-activation properties significantly different from those typical of slow- and fast-twitch fibres respectively (Stephenson & Forrest, 1980. See also Table 3). Therefore, it was desirable to use individual preparations isolated from the same animal and activated in the same batch of solutions to construct typical Ca-activation curves for the fast- and slow-twitch muscle fibres for comparative purposes. An averaging procedure for *all* relative force results at given pCa would hide essential information, particularly regarding the stoichiometry of Ca^{2+} ions in the process of muscle-fibre activation, due to the highly non-linear relationship between isometric force and pCa (Moisescu & Thieleczek, 1979). On the other hand, by choosing only one pair of activation curves from the total number of experiments at each temperature (seven at 3–5 °C; six at 22–25 °C; five at 35 °C) information would be lost regarding the biological variability in the Ca^{2+} sensitivity between individual rats. We have therefore decided to present our results for each temperature first by showing typical Ca-activation curves from individual preparations of the same animal activated in the same batch of solutions, and secondly by indicating the Ca^{2+} concentration range corresponding to 50% maximum force response (P_{50}^{Ca} (pCa)) at that temperature in our preparations.

Ca^{2+} activation at low temperatures (3–5 °C)

In Fig. 2 are illustrated individual force responses of one typical soleus and one typical e.d.l. preparation following activation in different Ca^{2+}-buffered solutions of type A/B (Table 1). The experiment was conducted in a thermostatically controlled

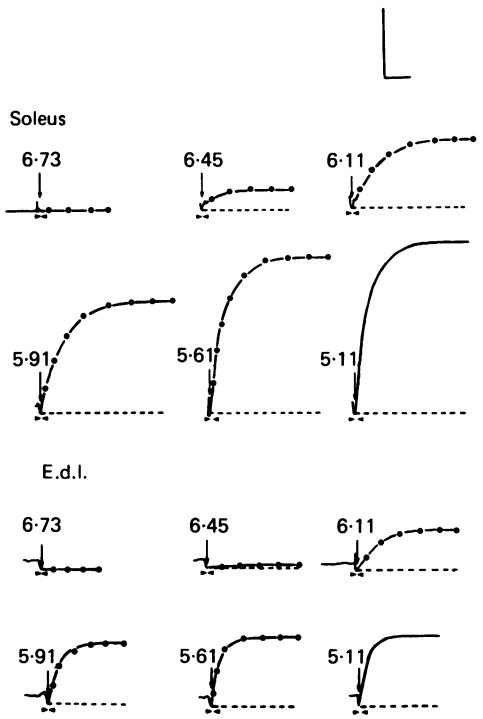

Fig. 2. Force responses from a typical slow-twitch (soleus) and a typical fast-twitch (e.d.l.) skinned muscle fibre of the same rat at 5 °C following rapid changes in [Ca^{2+}]. The preparations were first equilibrated for 3 min in a relaxing solution B, then transferred for 3 min into a low [EGTA] relaxing solution B/H (1/100) and finally activated (vertical arrows) in various solutions of type *A/B* (pCa indicated above vertical arrows). The distance between the horizontal arrows under each trace shows time intervals required for average Ca^{2+} within preparations to change to within 99 % of the Ca^{2+} in the bulk of the activating solutions (see text). The dots represent points taken from the predicted time course of relative force calculated using the models described in the text for stepwise Ca^{2+} changes from pCa 9 to the respective pCa. Calibration bars: horizontal 5s, vertical 0·1 mN. Dimensions of preparations (length, diameter, sarcomere length): soleus (1·2 mm, 50 μm, 2·76 μm) and e.d.l. (1·0 mm, 38 μm, 2·75 μm).

cold room at 5 ± 0·1 °C using the activation procedure of Moisescu & Thieleczek (1978) which enables a rapid equilibration of Ca^{2+} within the whole cross-section of the skinned muscle fibre (Moisescu, 1976; Griffiths, Kuhn, Güth & Rüegg, 1979).

Assuming a value of 4×10^{-6} $cm^2 . s^{-1}$ for the diffusion coefficient of $EGTA^{2-}$ and Ca $EGTA^{2-}$ in myofibrillar preparations at 5 °C (Moisescu & Thieleczek, 1978), we have estimated the time intervals required for the average Ca^{2+} concentration within our preparation to reach at least 99 % of its value in the activating solutions following

immersion. These time intervals are indicated as the distance between the two horizontal arrows under each trace in Fig. 2. Based on these results, we considered that the force responses in the e.d.l. and soleus preparations could be assumed to be the result of stepwise changes in Ca^{2+} concentration over the pCa range 6·80–5·60.

The time required to reach 50 % of the steady-state force level (t_{50}) decreased consistently in the e.d.l. preparation as Ca^{2+} concentration increased. A similar

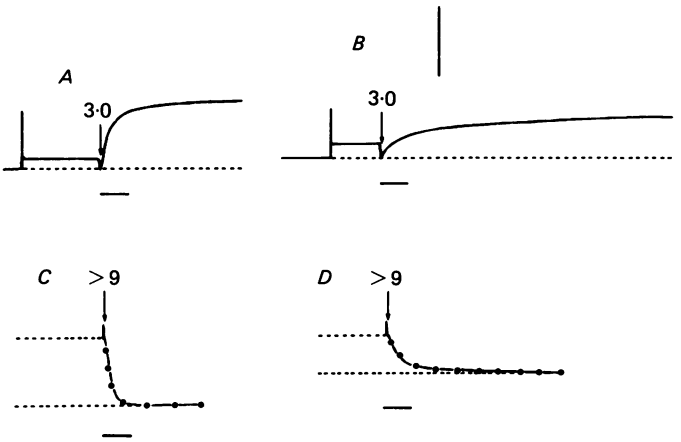

Fig. 3. Rapid maximal Ca^{2+} activation (*A*, *B*) and relaxation (*C*, *D*) responses in typical e.d.l. (*A*, *C*) and soleus (*B*, *D*) fibres from the same rat at 5 °C. In *A* and *B* the preparations were initially equilibrated in a relaxing solution of type NB and then activated in a solution of type NA in which Ca^{2+} was buffered to 1 mM. In *C* and *D* the preparations were first maximally activated in an unbuffered Ca^{2+} solution H (pCa $\simeq$ 4·5) and then suddenly relaxed in solution B. Estimated time for Ca^{2+} in any part of the preparations to decrease to below 10^{-8} M was less than 40 ms. Relaxation traces *C* and *D* are fitted by points taken from the predicted time course of relative force calculated using the models described in the text for a stepwise decrease in Ca^{2+} to pCa > 8. Calibration bars: horizontal 2s *A*, *B*; 1s *C*; 3s *D*. vertical 0·1 mN. Dimensions (length, diameter, sarcomere length): 1 mm, 36 μm, 2·85 μm for e.d.l. and 0·8 mm, 28 μm, 2·8 μm for soleus preparations respectively.

observation has been made on skinned muscle fibres of frog (Moisescu, 1976). However the typical soleus preparation showed an essentially different trend: the t_{50} values for steady-state forces below 15 % P_0 were relatively short and they gradually increased until [Ca^{2+}]s corresponding to about 40 % P_0 were reached. Only thereafter did t_{50} uniformly and continuously decrease with an increase in [Ca^{2+}].

Force response reached the steady-state level considerably faster in e.d.l. than in soleus preparations for the pCa range 6·00–5·10 corresponding to forces above 50 % in the soleus and above 70 % in the e.d.l. muscle fibres. However force developed faster in soleus than in e.d.l. preparations for $Ca^{2+} < 6 \times 10^{-7}$ M.

In Fig. 3 force responses are also shown following a rapid Ca^{2+} increase to 1 mM using a calcium buffer with a high buffering capacity around pCa 3 to allow fast [Ca^{2+}] equilibration in this range (Moisescu, 1976). One can see that force develops faster than in the traces shown for pCa 5·11 in Fig. 2, with half-time values closer to those observed *in situ* for tetanus, 0·15 s for e.d.l. and 0·6 s for soleus muscles (I. R. Wendt,

unpublished). These results indicate that: (i) rate of force development increases further with an increase in Ca^{2+} concentration beyond the pCa range for which a step-wise change in Ca^{2+} can be assumed and (ii) force develops more rapidly in fast-twitch muscle fibres than in slow-twitch muscle fibres (by at least a factor of 2) at Ca^{2+} concentrations expected to be reached during tetanus.

We also investigated the time course of force relaxation following a sudden drop in Ca^{2+} concentration. A rapid decrease in Ca^{2+} can be obtained, for example, by relaxing the preparation in a solution of type B, containing 50 mM-EGTA after full activation in a solution of type H containing about 3×10^{-5} M unbuffered Ca^{2+} (Moisescu & Thieleczek, 1978). In Fig. 3 relaxation curves are shown for typical e.d.l. and soleus muscle fibres which can be well fitted with simple exponential functions with rate constants of 0·67 s^{-1} and 4·4 s^{-1} for the soleus and e.d.l. fibres respectively.

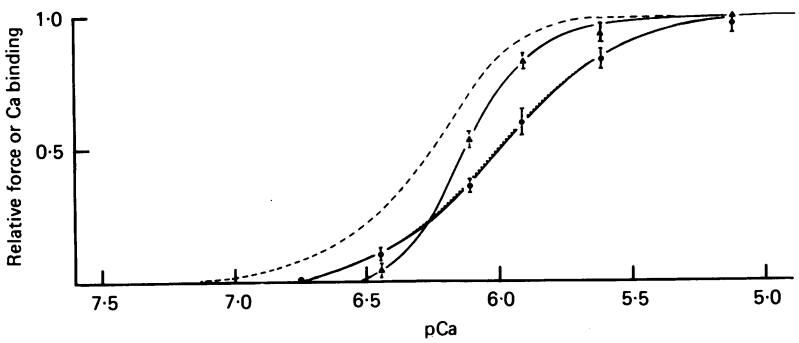

Fig. 4. Typical relative steady-state force–pCa relations at 5 °C in the same fast (—▲—) and slow-twitch (—●—) skinned muscle fibres as in Fig. 2, and for the same experimental conditions. The continuous lines are the predicted theoretical curves for relative force based on the models presented in the text and the dotted lines represent the predictions for the relative Ca-binding curves of the myofibrils for the fast-twitch (– – –) and slow-twitch muscle fibres (. . . .) respectively. The absolute scatter of the results is indicated by the vertical bars.

These curves are very similar to the relaxation curves obtained *in situ* for similar conditions (Hill, 1972; I. R. Wendt, unpublished) and strongly suggest that the rate of relaxation *in vivo* could be determined in large part by the properties of the contractile apparatus rather than those of the sarcoplasmic reticulum.

The steady-state Ca^{2+}-activation curves derived from the results in Fig. 2 are presented in Fig. 4, and it can be seen that the fast fibre shows a steeper curve relating force to Ca^{2+} concentration than the slow fibre. The apparent Ca^{2+} threshold for activation is significantly lower for the slow fibre, but force reaches 20 % P_0 in both fibre types at similar Ca^{2+} concentrations.

The summary of all results obtained at low temperatures with all preparations is shown in Table 3. From these results it is apparent that there is a variation in Ca^{2+} sensitivity associated with the contractile apparatus of about 0·25 pCa units for the fast type and 0·37 pCa units for the slow type of rat muscle fibres. The lower Ca^{2+} threshold in slow-type muscle fibres is also apparent from the summarized results in Table 3, although it is not as obvious as when using paired preparations from the same animal (Fig. 4).

TABLE 3. Comparative parameters for Ca^{2+}-activation in soleus and e.d.l. muscle fibres

Muscle type	Temperature (°C)	Sarcomere length (μm)	No. of preparations (animals) used	P_{50}^{Ca} (pCa) Range	P_{50}^{Ca} (pCa) Mean	ΔpCa (10–90 %) Range	ΔpCa (10–90 %) Mean	n*
Typical soleus	3–5	2·7–2·8	7 (6)	6·01–6·38	6·23	0·91–1·25	1·05	2
Atypical soleus	5	2·7	1 (1)	—	6·01	—	0·77	3
Typical soleus	22–25	2·65–2·75	8 (6)	5·85–6·60	6·22	0·89–1·01	0·95	2
Typical soleus	35	2·65–2·76	5 (4)	6·03–6·12	6·06	0·91–1·01	0·95	2
Atypical soleus	35	2·73	1 (1)	—	6·24	—	0·64	3
Typical e.d.l.	3–5	2·7–2·8	8 (5)	6·11–6·36	6·26	0·40–0·52	0·47	4
Typical e.d.l.	22–25	2·6–2·75	6 (6)	5·86–6·05	5·93	0·45–0·50	0·47	4
Typical e.d.l.	35	2·6–2·76	7 (5)	5·81–6·02	5·93	0·40–0·55	0·47	4

*n is the closest integer value for the Hill Coefficient from equation $P_r = K[Ca]^n/(1 + K[Ca]^n)$ where P_r represents the fraction of the maximum force response P_0, and K is a constant.

Ca^{2+} kinetics in soleus and e.d.l. muscle fibres

Further quantitative information regarding the process of Ca activation in mammalian muscle may be derived from simple kinetic schemes of reaction which predict accurately all force results obtained with each type of muscle fibre from the time course of Ca^{2+} concentration changes (Ashley & Moisescu, 1972; 1977; Moisescu, 1976).

From the steepness of the steady-state isometric force–pCa curves, and assuming over-all reversible Ca^{2+} reactions, one may estimate the minimum number m, of Ca^{2+} ions cooperatively involved in the process of force regulation (Moisescu, Ashley & Campbell, 1975; Moisescu, 1976; Miller & Moisescu, 1976). Any co-operative interaction between subunits of the functional regulatory unit which can influence the binding of Ca^{2+} ions and thus the mechanism of force regulation will be reflected in the absolute value of m. This number m may also be regarded as corresponding to the lowest integer satisfying the condition $m \geqslant n$ where n is the Hill Coefficient shown in Table 3. Based on the results shown in Fig. 4 (see also Stephenson & Forrest, 1980) and Table 3 it appears that m must be at least 2 in typical soleus and at least 4 in typical e.d.l. muscle fibres.

From Table 3, one can see that there were soleus fibres which had a Hill coefficient of 3. The time course of force development in these atypical soleus preparations more closely resembled that of the e.d.l. preparations than of the rest of the soleus preparations and it is reasonable to assume that these might have been representative of the small group (10–15%) of red, fast-twitch fibres present in rat soleus (Close, 1972; Table 2).

However, those results where the force responses can be regarded as resulting from stepwise changes in $[Ca^{2+}]$ are most relevant for such a kinetic analysis. In this analysis it was assumed that the kinetics of Ca^{2+} activation as reflected by the time course of the force responses in Fig. 2 for $pCa > 5{\cdot}6$, were much slower than the kinetics of force-generating complexes such as cross-bridges. This assumption appears reasonable considering the results in Fig. 3*A* and *B* where force is shown to develop significantly faster at higher Ca^{2+} concentrations than in Fig. 2, and preliminary quick-release experiments with mammalian preparations, which also indicate that the slowest rate of force recovery following release is much faster than the fastest rate of force development shown in Fig. 2 (D. G. Stephenson & D. A. Williams, unpublished).

The simplest scheme of reaction (in terms of minimum number of Ca^{2+} ions and minimum number of intermediary reaction steps involved) which can accurately explain the force–response curves obtained with the slow-twitch muscle fibre type can be written as follows:

$$Ca + R_s \underset{k_{-1s}}{\overset{k_{1s}}{\rightleftharpoons}} CaR_s + Ca \underset{k_{-2s}}{\overset{k_{2s}}{\rightleftharpoons}} Ca_2R_s \propto P_{rs}, \tag{2}$$

where R_s is the regulatory unit in the slow-twitch muscle fibre, k_{1s}, k_{-1s}, k_{2s}, k_{-2s} are the rate constants and P_{rs} is the isometric force response expressed as a fraction of the maximal isometric force P_0. The value of k_{-2s} is given by the relaxation rate in Fig. 3*D* and the values for the other rate constants were adjusted to obtain the best fit for the soleus results in Figs. 2 and 3. The dots in Fig. 2 and the continuous

curve in Fig. 4 which are overlaid on the experimental results represent the predictions from this scheme with the following values for the rates of reaction:

$$k_{1s} = 1{\cdot}07 \,.\, 10^5 \text{ M}^{-1} \text{ s}^{-1};\ k_{2s} = 2{\cdot}5 \,.\, 10^7 \text{ M}^{-1} \text{ s}^{-1};\ k_{-1s} = 4{\cdot}2\text{s}^{-1};\ k_{-2s} = 0{\cdot}67\text{s}^{-1}.$$

Fig. 4 also shows the predicted curve for the relative Ca binding on the force regulatory sites against pCa. This is very similar to the Ca^{2+}-activation curve for force and located only slightly to higher pCa values.

In the case of e.d.l. fibres it is noteworthy that kinetic schemes involving less than 6 Ca^{2+} ions per functional regulatory unit cannot adequately explain the time course of force responses following sudden changes in Ca^{2+} concentration. The results can however be explained accurately by the same type of kinetic scheme of reaction which was developed for amphibian twitch muscle (Moisescu, 1976):

$$\underset{\text{v. fast}}{\overset{k_{1f}}{\underset{k_{-1f}}{\rightleftharpoons}}} \qquad \underset{\text{v. fast}}{\overset{k_{2f}}{\underset{k_{-2f}}{\rightleftharpoons}}} \qquad \underset{\text{v. fast}}{\overset{k_{3f}}{\underset{k_{-3f}}{\rightleftharpoons}}} \qquad \underset{\text{slow}}{\overset{k_{4f}}{\underset{k_{-4f}}{\rightleftharpoons}}} \tag{3a}$$

$$\mathrm{Ca} + \mathrm{R_{1f}} \rightleftharpoons \mathrm{CaR_{1f}} + \mathrm{Ca} \rightleftharpoons \mathrm{Ca_2R_{1f}} + \mathrm{Ca} \rightleftharpoons \mathrm{Ca_3R_{1f}} + \mathrm{Ca} \rightleftharpoons \mathrm{Ca_4R_{1f}}$$

$$\underset{\text{fast}}{\overset{k_{5f}}{\underset{k_{-5f}}{\rightleftharpoons}}} \qquad \underset{\text{fast}}{\overset{k_{6f}}{\underset{k_{-6f}}{\rightleftharpoons}}} \tag{3b}$$

$$\mathrm{Ca} + \mathrm{R_{2f}} \rightleftharpoons \mathrm{CaR_{2f}} + \mathrm{Ca} \rightleftharpoons \mathrm{Ca_2R_{2f}}$$

The relative isometric force of fast-twitch fibres, P_{rf}, is considered to be proportional to the product of reaction $Ca_4R_{1f} - Ca_2R_{2f}$. ($R_{1f} - R_{2f}$) respresents the functional regulatory unit in fast muscle; k_{if}, k_{-if} and $K_{if} = k_{if}/k_{-if}$, (where $i = 1$–6) are the respective rate and equilibrium constants.

The value of k_{-6} was suggested by the rate of force relaxation in Fig. 3*C* and k_{-4} was chosen so as to correspond to the slowest rate of force rise in Fig. 2 for the e.d.l. fibre. The dependence of isometric force on the algebraic product between the concentration of Ca_4R_{1f} and that of Ca_2R_{2f} (each resulting from independent reactions) is important in explaining the e.d.l. force traces in Figs. 2 and 3. Thus the rate of force development following Ca^{2+} activation is controlled by the concentration term reaching the steady state more slowly (i.e. $[Ca_4R_{1f}]$) while the rate of relaxation following the removal of Ca^{2+} is mainly controlled by the fastest disappearing product of reaction (i.e. Ca_2R_{2f}).

The formation rates of all Ca-containing products in reaction schemes (3*a*) and (3*b*) are directly proportional to Ca^{2+} concentration and this results in an increased over-all rate for the equilibration of force at higher Ca^{2+}. All e.d.l. force results in Fig. 2 may be fitted reasonably well with simple exponentials and this is equivalent to assuming only one slowly equilibrating step of reaction in the whole set of reactions for e.d.l. Theoretical curves for e.d.l. force responses were calculated from the schemes of reaction in eqns. (3*a*) and (3*b*) using the following set of values for the rate and equilibrium constants: $k_{-4f} = 0{\cdot}2\,\text{s}^{-1}$; k_{-1f}, k_{-2f}, $k_{-3f} > 15\,\text{s}^{-1}$; $K_{1f} \,.\, K_{2f} = 3{\cdot}10^{11}\,\text{M}^{-2}$; $K_{1f} < 10^{-3} \times K_{2f}$; $K_{3f}\,K_{4f} = 1{\cdot}83 \times 10^{13}\,\text{M}^{-2}$; $K_{1f}\,K_{2f}\,K_{3f} < 1{\cdot}3 \times 10^6\,\text{M}^{-3}$; $k_{5f} = 2{\cdot}33 \times 10^5\,\text{M}^{-1}\,\text{s}^{-1}$; $k_{-5f} = 7\,\text{s}^{-1}$; $k_{6f} = 1{\cdot}23 \times 10^9\,\text{M}^{-1}\,\text{s}^{-1}$; $k_{-6f} = 4{\cdot}4\,\text{s}^{-1}$. The theoretical curves are overlaid on the experimental results in Figs. 2, 3 and 4 and the rather close agreement between the predicted and experimental results can be seen. Fig. 4 also shows the predicted curve for relative steady-state Ca binding to the regulatory system, which is significantly different from the force steady-state curve. The

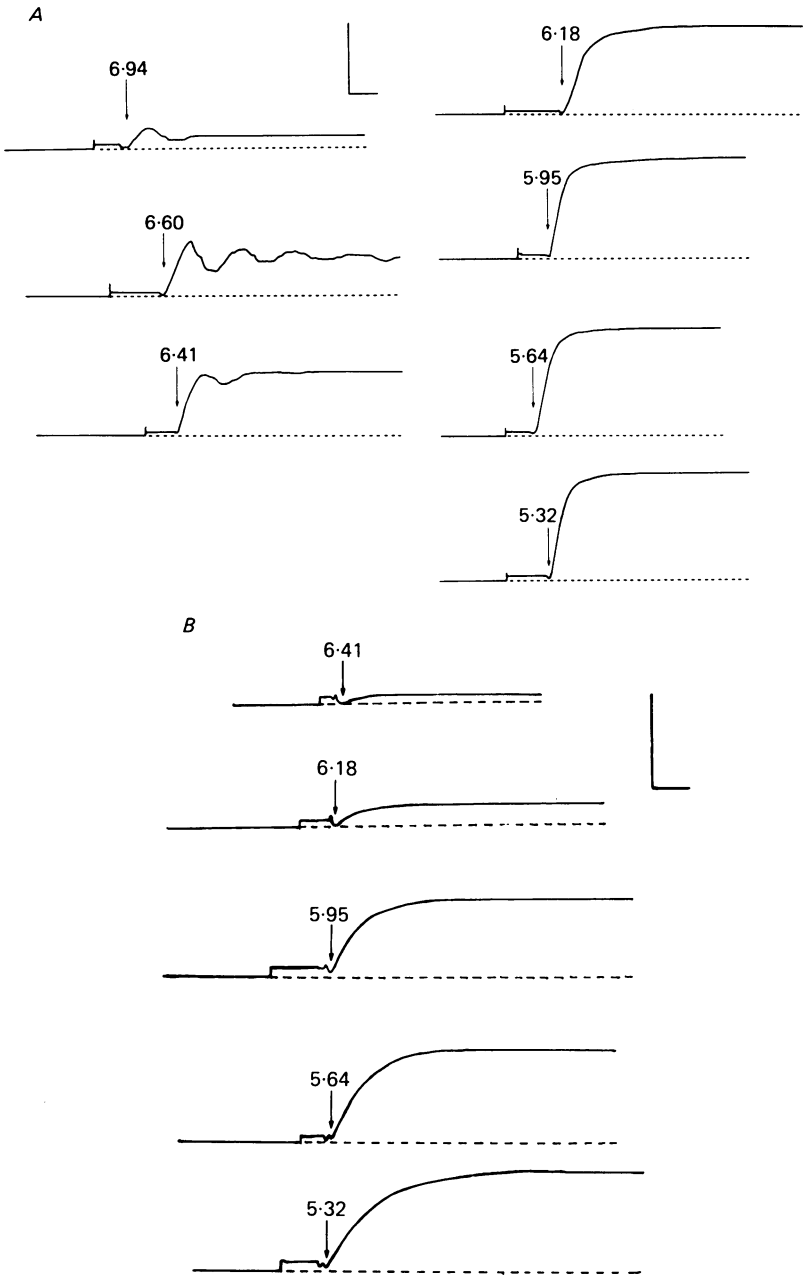

Fig. 5. Force responses from *A*, typical soleus and *B*, typical e.d.l. skinned muscle fibres of the same rat at 22 °C in solutions of type H′/A′/B′ following rapid changes in [Ca^{2+}] (see Fig. 2). Calibration bars: horizontal 1 s, vertical 0·25 mN. Dimensions of preparations (length, diameter, sarcomere length): *A* (1·0 mm, 45 μm, 2·7 μm) and *B* (0·9 mm, 35 μm, 2·8 μm).

mathematical expressions used to calculate all the theoretical curves were derived from the kinetic schemes for soleus and e.d.l. in the manner described by Rodiguin & Rodiguina (1964).

Ca^{2+} activation at room (22–25 °C) and high (35 °C) temperature

Individual responses from typical slow- and fast-twitch skinned muscle fibres suddenly activated at room temperature and 35 °C in Ca^{2+}-buffered solutions of type A′ or B′ and A″ or B″ are presented in Figs. 5 and 6 respectively. The rate of force

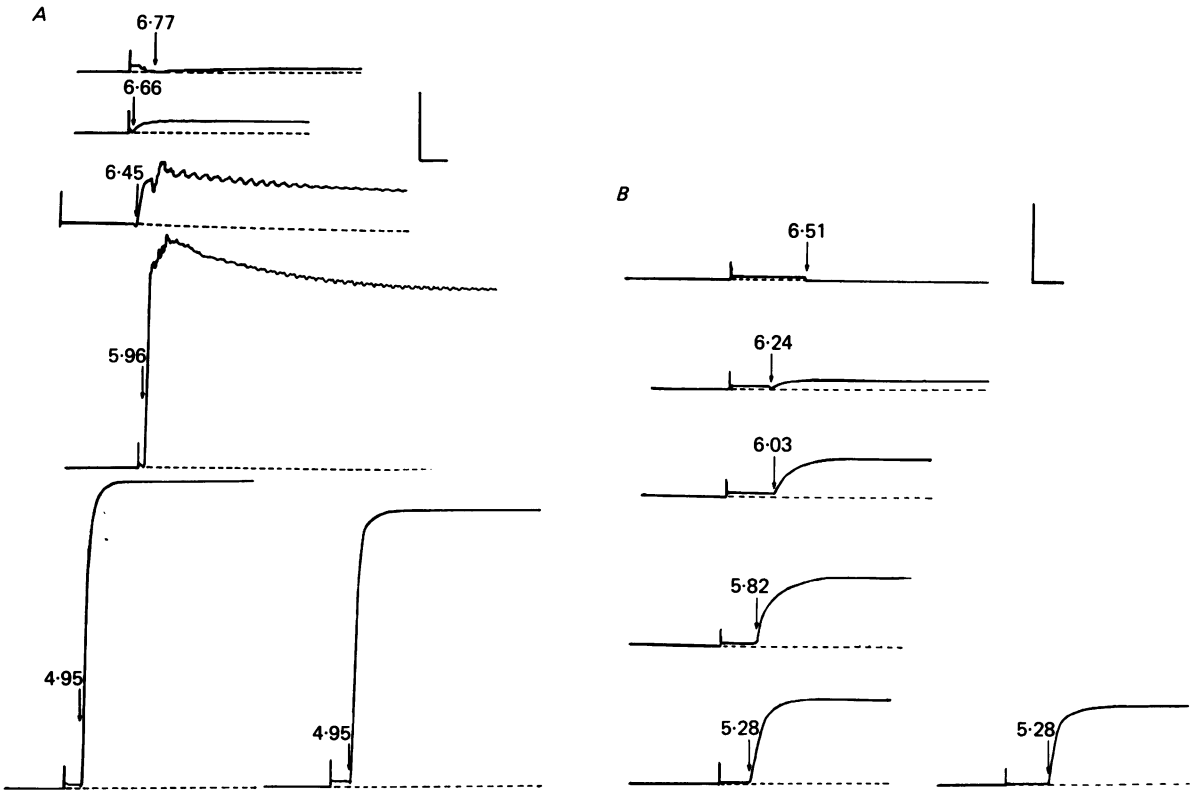

Fig. 6. Force responses from a typical soleus (*A*) and e.d.l. (*B*) skinned muscle fibre of rat at 35 °C in solutions of type H″/A″/B″ following rapid changes in Ca^{2+} (see Fig. 2). The last traces in both *A* and *B* represent consecutive maximum contractions between which five submaximal contractions were performed. Calibration bars: horizontal 1 s; vertical (*A*) 0·1 mN, (*B*) 0·2 mN. Dimensions of preparations (length, diameter, sarcomere length): *A* (2 mm, 50 μm, 2·7 μm) and *B* (1·25 mm, 30 μm, 2·7 μm).

development in both e.d.l. and soleus preparations increased markedly with an increase in temperature between 5° and 35 °C. One can estimate that the value of t_{50} for similar relative force responses (expressed as % of P_0) decreased by about a factor of 4 for soleus and 2 for e.d.l. preparations when the temperature increased by 17 °C, from 5 to 22 °C (cf. Figs. 2 and 5). In typical e.d.l. fibres force equilibrates even faster at 35 °C than at room temperature. This clearly indicates that a simple physical process, such as diffusion of Ca^{2+} within the preparations, was not rate-limiting for

force development in the experiments at 3–5 °C. Otherwise, the observed rate of force development would have been highly dependent upon the diameter of the preparation and only slightly dependent on temperature. However, diffusion is expected to become a limiting factor at 25° and 35 °C.

As can be seen from Fig. 6 the absolute maximum force response in these mammalian muscle preparations did not show a pronounced decline between successive maximum contractions in our experiments, even at these relatively high temperatures. This suggests that the contractile apparatus did not suffer extensive irreversible damage during the process of activation. However,

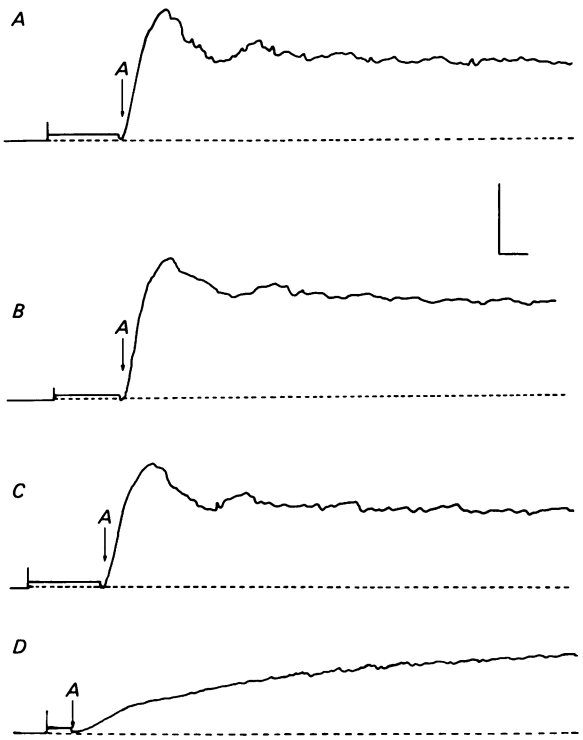

Fig. 7. Force oscillations induced in a typical skinned soleus muscle fibre partially activated ($\simeq 50\,\%\ P_0$) in a solution of type A′/B′ (pCa 6·04) before (*A*) and after being incubated for 10 (*B*) and 20 min (*C*) respectively, in a solution of type B′ containing 2 % v/v Triton X-100. In *D* Ca^{2+} within the preparation was changed much more slowly following activation in a solution of type H′/A′/B′ (pCa 6·04, total EGTA 2 mM) after being initially equilibrated in a solution H′/B′ (1/100). Calibration bars: horizontal 1 s, vertical 0·2 mN. Dimensions (length, diameter, sarcomere length): 1 mm, 45 μm, 2·8 μm.

it is important to note that prolonged, high levels of activation at 25 °C and 35 °C did result in significant residual force following relaxation in solutions with Ca^{2+} below 10^{-9} M. This could be associated with a partial loss of the regulatory control of contraction by Ca^{2+}. Similar observations were made on amphibian skinned muscle fibres by Thames, Teichholz & Podolsky (1974). Such residual forces were never observed in our experiment at 5 °C, even when the preparations were fully activated for several minutes. This again emphasizes the importance of employing methods which produce sudden changes in Ca^{2+} concentrations in skinned muscle preparations, especially at temperatures higher than 20 °C.

A striking feature of the force traces for soleus at and above room temperature is the presence of characteristic oscillations at Ca^{2+} concentrations corresponding to steady-state force levels between 10 and 60 % P_0. From our experimental records two types of oscillatory processes may be distinguished: a low frequency, high amplitude, damped, sinusoidal type oscillation immediately following activation and a high

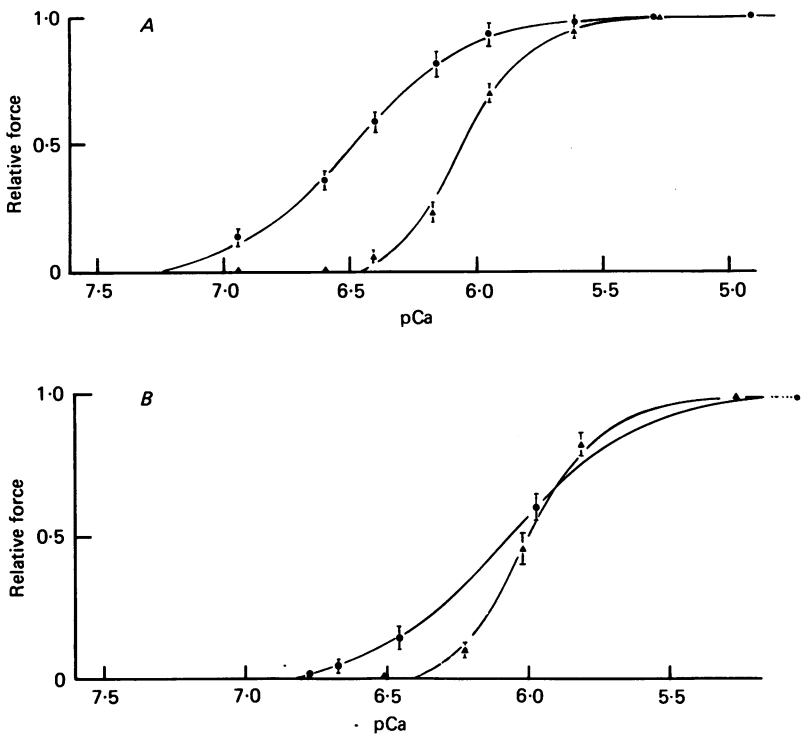

Fig. 8. Relative steady-state force–pCa relations at 22 °C (*A*) and 35 °C (*B*) in the same e.d.l. (—▲—) and soleus (—●—) skinned muscle fibres as shown in Figs. 5 and 6 respectively. The lines represent theoretical predictions from kinetic schemes (2) and (3) for soleus and e.d.l. respectively using the following parameters: (*A*) $k_{1s}k_{2s}/k_{-1s}k_{-2s} = 1 \times 10^{13}$ M^{-2}; $k_{1s}/k_{-1s} < 10^{-3}k_{2s}/k_{-2s}$; (*B*) $k_{1f}k_{2f}/k_{-1f}k_{-2f} = 1{\cdot}69 \times 10^{11}$ M^{-2}; $k_{1f}/k_{-1f} < 10^{-3}k_{2f}/k_{-2f}$; $k_{1f}k_{2f}k_{3f}/k_{-1f}k_{-2f}k_{-3f} < 5{\cdot}5 \times 10^{5}$ M^{-3}; $k_{3f}k_{4f}/k_{-3f}k_{-4f} = 1{\cdot}03 \times 10^{13}$ M^{-2}; $k_{5f}k_{6f}/k_{-5f}k_{-6f} = 5{\cdot}25 \times 10^{12}$ M^{-2}; $k_{5f}/k_{-5f} < 10^{-3}k_{6f}/f_{-6f}$.

frequency, small amplitude, undamped oscillation of irregular configuration (see also Fig. 7). Such oscillations were never observed in any e.d.l. fibres under any experimental conditions. Treatment of the skinned fibres with detergents which are known to disrupt and remove intracellular membranes (Fig. 7) did not abolish or diminish the oscillatory responses. This strongly suggests that these oscillations were not associated with membranous intracellular compartments such as the sarcoplasmic reticulum and mitochondria. The large amplitude oscillations could be generated only at lower levels of activation, and only when the Ca^{2+} concentration was increased rapidly in the preparations as for figures 5*A* and 7*A–C*. If Ca^{2+} was increased more slowly (Fig. 7*D*) then the large amplitude oscillatory response was no longer

discernible, while the low amplitude, high frequency oscillations were not greatly affected.

Oscillatory processes were less accentuated and slightly more uniform at 35 °C than at room temperature. The frequency of both types of oscillations did not appear to change greatly with Ca^{2+} concentration but was affected by temperature. A rise in temperature from 25 to 35 °C increased the frequency from *ca.* 0·4 Hz to 2·3 Hz and from 4 to 10 Hz for the low and high frequency oscillatory processes respectively. A reduction in temperature to 5 °C resulted in the complete abolition of these oscillations.

The steady-state Ca^{2+}-activation curves derived from the force responses shown in Figs. 5 and 6 are plotted in Fig. 8. When oscillatory responses were present in soleus preparations the steady-state force was considered to be the average force level after the disappearance of the large amplitude oscillations. There is apparently a more pronounced difference in the Ca^{2+} sensitivity between the two fibre types at room temperature than at either low temperature or 35 °C. This is mainly due to the fact that the whole Ca^{2+}-activation curve presented for the soleus fibre at room temperature is shifted 0·5 pCa units toward lower $[Ca^{2+}]$ in comparison to those at 5 or 35 °C, while the Ca^{2+}-activation curves of the e.d.l. fibre type are only slightly shifted on the pCa axis. However, paired muscle fibres from other rats did not always show such a pronounced sensitivity difference between the Ca^{2+}-activation curves in slow- and fast-twitch muscle fibres at 22 °C. A summary of all results obtained at room temperature and 35 °C is included in Table 3.

Temperature effect on maximum force response

Intact rat muscle fibres, unlike intact amphibian muscle fibres, respond with greatly diminishing force to optimal tetanic stimulation when temperature is descreased below 20 °C (e.g. Cullingham *et al.* 1960; Truong *et al.* 1964; Isaacson, *et al.* 1970) and this has been mainly attributed to a blockage in the excitation–contraction coupling process at lower temperatures.

In order to determine to what extent this reduction in force response is due to an effect on the contractile apparatus itself, we performed a series of experiments in which the same muscle preparations were fully activated at different temperatures in solutions of otherwise similar composition (solutions A, A′, A″ Table 1) with $[Ca^{2+}]$ higher than 10^{-5} M.

Fig. 9 shows the average values for the maximum force per cross-sectional area in slow- and fast-twitch muscle preparations at different temperatures. It also shows the average of the ratios between P_0 in slow- and P_0 in fast-twitch muscle fibres from the same rats, indicating significantly higher tensions in e.d.l. for temperatures around 35 °C and lower tension in e.d.l. at 5 °C ($P < 0{\cdot}01$, d test). An initial observation is that the average maximum force response per cross-sectional area appears to be higher in our preparations (*ca.* 30 N/cm² for slow muscle fibres and 40 N/cm² for fast muscle fibres at 35 °C) than in whole muscles (25 N/cm² for soleus and 30 N/cm² for e.d.l. at 35 °C, Close & Hoh (1968)). From Fig. 9 it can also be seen that P_0 declined steeply to zero in both fibres when temperature was decreased from 20 to 1 °C, while increasing only marginally when temperature was increased from 25 to 35 °C. When using the same preparations at 25 and 35 °C, in order to minimize

errors in determining the relative force responses, one could observe a consistently higher maximal force at 35 than at 25 °C (10–12 % for slow fibre and 30 % for fast fibre; Stephenson & Williams, 1980*a*).

The observed differences in the maximum force response cannot be due to the small variation in osmolarity between the activating solutions at various temperatures since control experiments with added glucose (up to 100 mosM) did not reveal any significant differences in the level of maximum force.

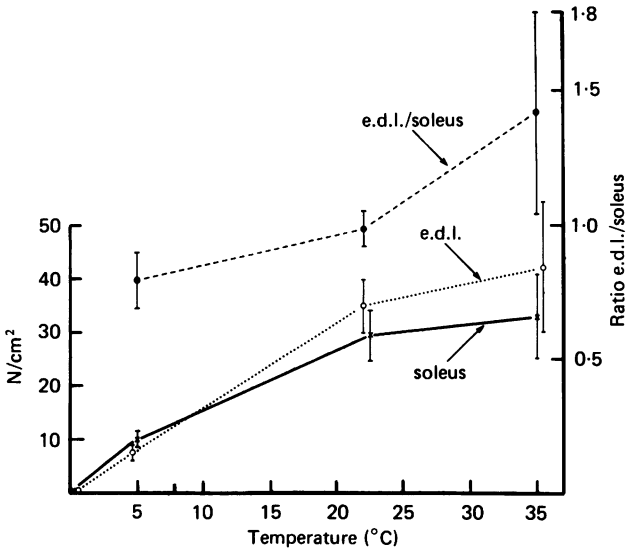

Fig. 9. Graphical representation of the average absolute force following maximal Ca^{2+} activation in typical e.d.l. (dotted line) and soleus (continuous line) preparations as a function of temperature. The average ratio between the maximal force response in e.d.l. and soleus preparations from the same rat is also shown (dashed line; right ordinate). The vertical bars indicate the S.D. of the results.

The strong temperature dependence of the maximum force in skeletal muscle fibres of mammals below 20 °C raises the rather important question as to whether this temperature dependence is related to associated changes in the ATPase activity of the contractile proteins and/or to changes in the maximum number of interacting sites between myosin and actin filaments. In an attempt to discover which of these possibilities is more likely some experiments on the rigor state were performed. These showed that: (i) rigor force levels decreased only marginally if the temperature was reduced from 22 to 5 °C after the state of rigor was initially induced at 22 °C, (ii) rigor force levels at 5 °C were about 15–20 times lower than those at 22 °C, irrespective of whether the rigor states were induced independently at each temperature or the temperature was increased after the rigor state was initially induced at 5 °C. If the rigor force level is assumed to be proportional to the number of interacting sites between the actin and myosin filaments, then result (i) can be interpreted to suggest that the number of rigor complexes (once formed) does not fall following a decrease in temperature and that the level of force per rigor complex does not vary greatly between 5 and 22 °C. The results from (ii) may then be interpreted as indirect

evidence for the contention that the number of rigor interactions between filaments is much reduced at low temperatures.

DISCUSSION

Results presented in this paper clearly show that there are characteristic relations between Ca^{2+} concentration and the steady-state isometric force response in mammalian fast- and slow-twitch muscle fibres at all temperatures investigated (3–35 °C). The increase in relative isometric force response from 10 % P_0 to 90 % P_0 is brought about by a threefold rise in Ca^{2+} concentration in the fast-twitch fibre while the slow-twitch fibre requires a tenfold rise to respond with an equivalent relative force increase. These characteristic relations are maintained in spite of the large variation in the absolute isometric force at different temperatures and the variation in Mg^{2+} concentration (0·1–1 mM; Stephenson & Forrest, 1980). The less steep force – pCa curves reported for skinned muscle fibres from two other mammals at room temperature (rabbit: Kerrick, Secrist, Coby & Lucas, 1976; Donaldson & Hermansen, 1978; and guinea-pig: Takagi & Endo, 1977) could be due to averaging results from preparations with significantly different Ca sensitivities (see Table 3 and Moisescu & Thieleczek, 1979).

A change in temperature from 3–5 °C to 22–25 °C has a significant effect ($P < 0{\cdot}01$, d test) on the sensitivity of the fast fibres to Ca^{2+}. The relative force–pCa curve was shifted, on average, by 0·33 pCa units towards lower pCa values. This decrease in Ca^{2+}-sensitivity with the increase in temperature did not however affect the steepness of the curves. A further increase in temperature from 22° to 35 °C did not produce any further significant change in the force–pCa relation. A similar statistical analysis of all Ca-activation curves in slow-twitch fibres showed no significant differences between force–pCa results at different temperatures, although the simple comparison of Fig. 4 and 8 might have suggested an increase in Ca^{2+} sensitivity following a temperature increase from 5° to 22 °C and a decrease in sensitivity following a temperature increase from 22° to 35 °C. This anomaly is due to the fact that the soleus fibres from different animals showed an unusually large variation in Ca^{2+} sensitivity at 22–25 °C (see Table 3). It is possible that skeletal muscle fibres from other mammals exhibit similar large variations in Ca^{2+} sensitivity at room temperature. If so, such a variability could account for previously reported apparently inconsistent differences between Ca^{2+} sensitivity in slow- and fast-twitch muscle fibres (Kerrick *et al.* 1976; Takagi & Endo, 1977).

All present results indicate that the Ca^{2+} threshold for activation is lower in slow-twitch than in fast-twitch muscle fibres of the same rat, and this is in agreement with other observations on skinned mammalian muscle fibres (Takagi & Endo, 1977; Kerrick *et al.* 1976). However, force does not necessarily saturate at lower Ca^{2+} concentrations in slow muscle fibres, and the physiological implications of this finding have been discussed previously (Stephenson & Forrest, 1980).

An important conclusion which can be drawn from these experiments is that differences in the properties of the myofibrillar component of the mammalian muscle fibres could be the main factor in determining the longer time to the peak of the twitch and the slower relaxation in slow-twitch muscle fibres (Close, 1972). Our results

indicate that force develops faster in fast-twitch than in slow-twitch muscle fibres at constant Ca^{2+} concentrations, corresponding to steady-state force levels higher than 40% P_0. Also the rate of force relaxation was smaller by a factor of 6 in the slow-type than in the fast-type preparation when Ca^{2+} concentration was rapidly decreased to subthreshold levels.

The rate of force development was shown to depend strongly upon the ionized calcium concentration and temperature in both preparations. The experimental results were adequately explained by two different reaction schemes for the activation of contraction by Ca^{2+}, and this suggests that the Ca-regulatory mechanism in the slow-twitch fibres is essentially different from that in the fast-twitch fibre. The reaction model for fast muscle fibre (eqns (3*a*) and (3*b*)) is interesting in that it predicts that: (i) the over-all rate of force development can increase to values similar to those observed *in vivo* providing that Ca^{2+} increases sufficiently rapidly above 10^{-5} M and (ii) the rate of relaxation of muscle is much faster than the dissociation rate for Ca^{2+} from one group of sites (k_{-4}) involved in regulation. This model shows that one cannot simply discard the involvement of the two high affinity Ca/Mg binding sites on troponin in Ca regulation, based solely on slower Ca-dissociation rates from these sites compared with the rate of relaxation of the muscle *in vivo* (Johnson, Charlton & Potter, 1979). Lowering Mg^{2+} from 1 mM to 0·1 mM under otherwise similar ionic conditions shifted the force–pCa curves towards lower [Ca^{2+}] (Fabiato & Fabiato, 1975; Ashley & Moisescu, 1974; Stephenson & Forrest, 1979, 1980) lending strong support to the hypothesis that all four Ca binding sites on troponin could be involved in the regulation of fast-twitch skeletal muscle. A simple involvement of all four Ca^{2+} ions on troponin in this process is not sufficient to account for the level of co-operative Ca^{2+} binding implicit in eqn. (3). However, independent biochemical observations have led Weber & Murray (1973) to suggest that the level of positive co-operativity between different Ca-binding subunits on the filaments could be increased in the presence of force-generating actomyosin interactions. Moreover our kinetic model in eqn. (3) requires two additional Ca^{2+} ions. One possible explanation is that the mammalian myosin light chains which were recently proved to restore Ca sensitivity to desensitized scallop myofibrils (Simmons & Szent-Gyorgyi,'1980) are also involved in the regulation of fast-twitch muscle fibres. Another possibility is that the Ca-dependent phosphorylation–dephosphorylation of myosin light chains also plays an important role in the regulation of this type of muscle (Frederiksen, 1980). A further possibility is that the actual regulatory functional unit in these muscles involves more than one troponin (Ashley & Moisescu, 1972). This is equivalent to assuming an over-all positive co-operativity between neighbouring Ca binding subunits of the same type. Regardless of their exact origin, the apparent large number of Ca^{2+} ions involved in the regulation of contraction of fast-twitch muscle enables a faster transition between the full activated and fully relaxed states which is physiologically important.

The two Ca^{2+} ions involved in the kinetic model for contractile regulation in slow-twitch muscle can be easily accounted for using biochemical observations on Ca binding to troponin (Ebashi & Endo, 1968).

An interesting prediction from our kinetic analysis is that the relative force –pCa curve closely follows the curve for relative Ca^{2+} binding to the regulatory sites in slow

twitch muscle (Fig. 4) but not in fast-twitch muscle where there is a large discrepancy between the Ca–force and Ca–binding curves. Biochemical observations on Ca-binding and ATPase activity (or force) in fast-twitch muscles fully support the latter part of this prediction (Weber, Herz & Reiss, 1964; Solaro, Wise, Shiner & Briggs, 1974).

From our results on maximum and rigor force response at different temperatures it appears that the total number of interacting sites between the myosin and actin filaments is dependent upon temperature, supporting the suggestion that for constant ionic conditions the affinity between myosin and actin decreases with a drop in temperature below 25 °C (Bendall, 1961). Above 25 °C the maximum force only slightly increases with temperature, suggesting that for our ionic conditions a saturation process (i.e. the maximum number of interactions between actin and myosin is reached) takes place within this range. This may be physiologically important when the range of temperatures over which the limb muscles in rat normally operate is considered.

A final aspect of this series of experiments relates to the consistent oscillatory processes which were observed only in the slow-twitch type of muscle fibres at 22–35 °C following submaximal Ca^{2+} activation. These oscillatory processes were not associated with intracellular membrane systems such as mitochondria and sarcoplasmic reticulum since: (i) all our solutions contained Na azide and caffeine which should have interfered with the Ca^{2+} movements associated with these compartments and (ii) additional treatment of the skinned muscle preparations with Triton X-100 did not abolish the oscillations (Fig. 7; and Williams & Stephenson, 1980). The oscillatory processes were most pronounced at Ca^{2+} concentration corresponding to about 50 % P_0 and could never be observed when the preparations were fully activated. Oscillations linked with the myofibrillar component have been reported for single mechanically skinned cardiac cells (Fabiato & Fabiato, 1978). Although physiological significance of these oscillations is not clear, they further underline the similarities between the contractile apparatus of cardiac and slow-twitch skeletal muscle. The explanation given by Fabiato & Fabiato (1978) for the generation of myofibrillar oscillations, namely that periodic shortening of some sarcomeres results in periodic stretch-induced increase in the Ca^{2+} sensitivity of other sarcomeres, could also be valid in principle for the high frequency oscillations. This is because we have noticed pronounced sarcomere length effects on the Ca^{2+}-activation curves, particularly in the slow-twitch fibre type (D. G. Stephenson & D. A. Williams, unpublished).

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