THERMAL DEPENDENCE OF MAXIMUM Ca^{2+}-ACTIVATED FORCE IN SKINNED MUSCLE FIBRES OF THE TOAD *BUFO MARINUS* ACCLIMATED AT DIFFERENT TEMPERATURES

BY B. B. REES* AND D. G. STEPHENSON

Department of Zoology, La Trobe University, Bundoora, Victoria, 3083 Australia

SUMMARY

1. Mechanically skinned muscle fibres from the twitch region of the iliofibularis muscle of cool- (16 ± 1°C) and warm- (32 ± 1°C) acclimated cane toads (*Bufo marinus*) were activated maximally by Ca^{2+} in solutions of different pH and at different temperatures (approx. 1–35°C). Acclimation of up to 12 weeks at 16°C and up to 8 weeks at 32°C did not modify the marked thermal dependence of isometric force in the skeletal muscle fibres of the cane toad.

2. The prominent decline of maximum Ca^{2+}-activated force at lower temperatures, a property which is not characteristic of muscles from other anurans, was associated with an obvious decline in fibre stiffness at temperatures below about 20°C, regardless of the temperatures at which the toads were kept prior to experimentation. The results suggest that the decline of isometric force at lower temperatures is due both to a reduction in the number of cross-bridges and to a decrease in the force output per cross-bridge.

3. The maximum Ca^{2+}-activated force response increased when fibres were activated in solutions of increasing pH at all temperatures investigated. This trend is expected to have a compensatory effect on the thermal dependence of the maximum Ca^{2+}-activated force under physiological conditions, because of the elevation of intracellular pH as temperature declines.

4. The isometric force did not depend on the concentration of the zwitterionic species of the pH buffer in solutions.

5. The skinned fibre preparation developed a Ca^{2+}-insensitive residual force following maximal activation. The increment in residual force followed a linear relationship with the duration of activation at a given temperature and a power relationship of activation temperature for a given duration of activation. Fibres from warm-acclimated animals developed less residual force following activations at 15°C than did fibres from cool-acclimated animals, suggesting that thermal acclimation may substantially reduce the magnitude of this phenomenon at temperatures below 20°C.

*Present address: Department of Environmental, Population and Organismic Biology, University of Colorado, Boulder, Colorado, USA.

INTRODUCTION

Maximum force production in skeletal muscle from temperate anurans is largely insensitive to change in environmental temperature. In both intact and skinned muscle fibre preparations this pattern of thermal independence is characterized by a plateau, extending over a range of 15–20°C, with forces greater than 80% of the highest force (for a review, see Bennett, 1984). This property of anuran skeletal muscle is directly related to the capacity of these animals to remain active over a broad range of ambient temperature. In a recent study of the cane toad, *Bufo marinus*, however, we showed that the maximum Ca^{2+}-activated force in skinned muscle fibres declined dramatically when the temperature was decreased from 20 to 0°C (Stephenson & Williams, 1985). This striking temperature dependence of force production is quite different from that previously described for anuran muscle, and it has been suggested that this pattern is related to the relatively constant thermal environment of the tropical habitat of *Bufo marinus*. Thus, we decided to characterize in more detail some basic properties of the contractile apparatus of skeletal muscle in this tropical anuran. In particular, we wondered whether there might be some compensatory modification of the capacity to generate isometric force as a result of short-term thermal acclimation. Previous studies with frog and toad muscles could not show such compensation (Renaud & Stevens, 1981a,b), but as tetanic force in these anurans was only slightly dependent upon temperature, this might have been expected.

In this study, muscle fibres dissected from the twitch region of the iliofibularis muscle were mechanically skinned to allow direct Ca^{2+}-activation of the contractile apparatus over a wide range of temperature. Since the pH of anuran sarcoplasm is known to increase with a decrease in temperature (Malan, Wilson & Reeves, 1976), fibres were activated over a range of pH values expected to reflect *in vivo* values at each temperature. The interactive effect of temperature and pH upon maximum force was assessed in fibres from toads acclimated to either cool or warm temperatures for up to 12 weeks. Furthermore, we performed stiffness measurements in an attempt to elucidate the mechanism responsible for the marked decline in force at lower temperatures and we studied in more detail the nature of the residual force which is known to develop in skinned muscle fibres following prolonged activation (Thames, Teichholz & Podolsky, 1974).

MATERIALS AND METHODS

Animals

Adult male and female cane toads, *Bufo marinus*, (80–200 g) were obtained from Queensland, Australia, from a local supplier. The animals were kept cool (16–17°C) after capture and then shipped by air to Melbourne. Toads were received in three shipments throughout the year and, upon arrival, divided into the two acclimation groups.

One group was maintained in a constant temperature room at 16 ± 1°C for up to 12 weeks on a layer of woodshavings kept moist by the occasional addition of water.

Toads received in the first shipment were provided with live crickets (two per toad) at 3-day intervals, but it was found that after the first week of maintenance toads did not feed, probably due to a decrease in metabolic rate. Subsequently no food was provided to the specimens at 16°C and these toads remained in good health and condition throughout the period of maintenance judging by their general appearance and by measurements of the thigh thickness.

The other group of toads was transferred to a constant temperature room at $32 \pm 1°C$. Toads received in the first shipment were kept on moistened wood-shavings and each fed two live crickets twice a week. After a few weeks we noticed significant weight loss in these specimens and accelerated decomposition of woodshavings at this temperature. Therefore, toads from subsequent shipments were fed 4–5 live crickets each, twice weekly and kept on a substrate of dampened paper towelling. A shallow container of water was also available to the animals. The towelling and the water were replaced twice weekly at the time of feeding. Toads maintained under this latter set of conditions did not lose weight and remained healthy, as judged by activity, appearance and measurements of the thigh thickness, for the duration of the acclimation period (up to 8 weeks). Unless otherwise mentioned, the observations regarding acclimation to warm conditions were made with muscle preparations obtained from the last two shipments of animals.

The cloacal temperatures of 10 toads from each group of acclimated animals were measured with a Jenway digital thermometer and thermocouple. The thermocouple was placed in the cloaca while the animal was held by the anterior part of the body and the temperature was recorded 15–20 s following placement of the thermocouple. Cloacal temperatures of the cool-acclimated toads ranged from 15·7 to 16·2°C and readings from warm-acclimated toads fell between 29·7 and 31·3°C, indicating that thermoregulation by toads under the conditions of acclimation studied was minimal.

The skinned muscle fibre preparation

Toads were killed by double pithing of the central nervous system. The iliofibularis muscle was dissected out and carefully blotted on Whatman (no. 1) analytical filter paper to remove excess interstitial fluid. The muscle was then immersed in paraffin oil (Ajax Chemicals, Sydney) contained in a Petri dish with a layer of transparent resin (Sylgard 184; Dow Corning, USA). Single fibres were isolated and then mechanically skinned under a dissecting microscope (Nikon, Japan), using jewellers' forceps and hypodermic needles as described in detail elsewhere (Ashley & Moisescu, 1977; Moisescu & Thieleczek, 1978; Stephenson & Williams, 1981). The skinning procedure was performed at room temperature and the unused sections were normally kept under paraffin oil at 5°C.

Apparatus, solutions and procedures

The skinned fibre preparations were mounted under oil either between an AE875 strain gauge force transducer (AME, Horten, Norway) and a pair of fine Barcroft forceps, as described by Stephenson & Williams (1981), or between a force transducing system based on the AE801 strain gauge (AME) and the arm of a

vibrator (Ling Dynamic Systems, V201, UK) which was driven by an LDS Power Oscillator (TPO25 UK). The AE801-based force transducing system comprised either a 35 mm glass tube (diameter 1·0 mm) which was in contact with the silicon beam of an AE801 strain gauge element (the tube was sealed at one end and encased at the other end in an aluminium block), or a stainless steel entomological pin (diameter 0·1 mm, length 4 mm) attached directly to the silicon beam with epoxy resin (Tra-Bond, BB-2126, Tra-Con, USA). The resonant frequencies of the force transducing systems were about 150 Hz and 2 kHz for the AE875 and AE801 systems, respectively. In a separate series of experiments we increased the resonant frequency of the AE801 force transducer to about 3 kHz by using a smaller pin attached with shellac to the silicon beam. In these experiments we measured the amplitude of the force oscillations induced by small length oscillations at 2·5 kHz with a precision absolute value converter built in the department's electronics workshop. The electrical signals from the strain gauge elements were displayed either on a chart recorder (Toshin Electron, Japan) or on a storage oscilloscope (Tektronix 5000, USA), and photographed.

Braided silk (Deknatel, 9·0, USA) was used to attach the preparations to the force transducing systems and to the arm of the vibrator as described by Julian, Rome, Stephenson & Striz (1986). The slack length and the apparent diameter of the skinned fibre were measured in oil under a dissecting microscope before immersing the preparation in a relaxing solution ($[Ca^{2+}] < 10^{-9}$ mol l^{-1}, pH 7·10 at 25 °C; for composition see below). The average sarcomere length was then determined in this relaxing solution from the diffraction pattern produced by a He–Ne laser (Spectra-Physics, 136-04, USA) as described by Stephenson & Williams (1981). Unless otherwise mentioned, the sarcomere length for these experiments was adjusted to between 2·16 and 2·30 μm.

The experimental solutions were of three types: high-EGTA [ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetraacetic acid] relaxing solutions (R), pre-activating solutions (PA) and maximally Ca^{2+}-activating solutions (A). All solutions contained in (mmol l^{-1}): K$^+$, 117; Na$^+$, 36; ATP$_{total}$, 8; Mg^{2+}, 1; creatine phosphate, 10; azide, 1. The endogenous creatine kinase of myofibrillar origin (Walliman, Turner & Eppenberger, 1977; Saks, Rosenstraukh, Smirnov & Chazov, 1978) in the skinned fibres was sufficient to maintain [MgATP] in the millimolar range throughout the fibres during activating, since addition of exogenous creatine phosphokinase (Sigma) up to 15 units ml^{-1} did not affect the results (see Results). The R solutions contained 50 mmol l^{-1} EGTA^{2-} with no added calcium ($[Ca^{2+}] < 10^{-9}$ mol l^{-1}), while the A solutions were carefully adjusted to contain Ca^{2+} and EGTA in equimolar (50 mmol l^{-1}) amounts (Stephenson & Williams, 1981). The ionized Ca^{2+} concentration in the activation solutions was between 2×10^{-5} and 10^{-4} mol l^{-1}, which ensured the full activation of the contractile apparatus over the entire range of conditions investigated (see also Stephenson & Williams, 1985). The main anionic species in the PA solutions was 49·80 mmol l^{-1} HDTA^{2-} (1,6-diaminohexane-N,N,N',N'-tetraacetic acid). The PA solution also contained 0·20 mmol l^{-1}

$EGTA^{2-}$, which maintained the $[Ca^{2+}]$ below the level of activation. This solution was used in conjunction with the 'Ca-jump' technique for the rapid activation of the skinned fibres (Moisescu, 1973, 1976; Ashley & Moisescu, 1973; Moisescu & Thieleczek, 1978; Stephenson & Williams, 1981). The technique involves equilibration of the skinned fibre preparation in the *PA* solution prior to activation in the *A* solutions.

In most experiments, we used four groups of solutions (one *R*, one *PA* and one *A* solution per group) which differed mainly in the concentration of the pH buffer, and pH. The first group of solutions contained 45 mmol l^{-1} of the pH buffer TES [*N*-tris-(hydroxymethyl)methyl-2-aminoethane sulphonic acid] and had a pH of 7·10 at 35 °C (pH 7·30 at 25 °C). The second group of solutions contained 60 mmol l^{-1} TES and had a pH of 7·10 at 25 °C. The third group contained 85 mmol l^{-1} TES and had a pH of 7·10 at 12 °C (pH 6·84 at 25 °C), and the fourth group contained 140 mmol l^{-1} TES and had a pH of 7·10 at 2 °C (pH 6·65 at 25 °C). The exact preparation and composition of the first, second and fourth sets of solutions has been described in detail elsewhere (Stephenson & Williams, 1981). The third group of solutions had the same cationic concentrations as the other groups and was prepared similarly (Stephenson & Williams, 1981). pH was the only parameter of physiological importance which changed significantly with the temperature of the activating solutions, because of the use of TES as the main pH buffer in these solutions ($\Delta pK = -0.02$ pH units °C^{-1}: Good *et al.* 1966). In separate experiments, we replaced the TES buffer with 11·5 mmol l^{-1} of the pH buffer, PIPES [piperazine-*N*,*N*'-bis(2-ethanesulphonic acid)] and added 15 units ml^{-1} creatine phosphokinase to all solutions. This pH buffer has a pK value of 6·80 at 20 °C and is rather insensitive to changes in temperature ($\Delta pK = 0.0085$ pH units °C^{-1}: Good *et al.* 1966). The pH was adjusted to 7·10 ± 0·01 in the four sets of solutions at 5°, 15°, 25° and 35 °C without changing the ionic strength between solutions by more than about 2 mmol l^{-1}.

The main chamber for skinned fibres consisted of 10 water-jacketed baths which could hold up to 4 ml of solution each. The temperature of the solutions was controlled to within ±0·3 °C of a particular temperature in a single series of activations and ±1 °C between experiments. The whole chamber could be moved within 2 s to change the solution bathing the preparation (for other details see Moisescu & Thieleczek, 1978).

In addition to the solutions in the main chamber (three or four groups of *R*, *PA* and *A* solutions), we also used another block with three solutions at room temperature (*R*, *PA*, *A* with 60 mmol l^{-1} TES), which could be transposed within a few seconds into position under the preparation. The skinned fibres were normally bathed in the *R* solution (60 mmol l^{-1} TES) at room temperature while changing the temperature of the solutions in the main chamber. During an experiment the preparations were activated at regular intervals in the *A* solution at room temperature in order to determine the extent of fibre deterioration during the course of an experiment. In a single experiment, all responses were initially standardized to the response at room
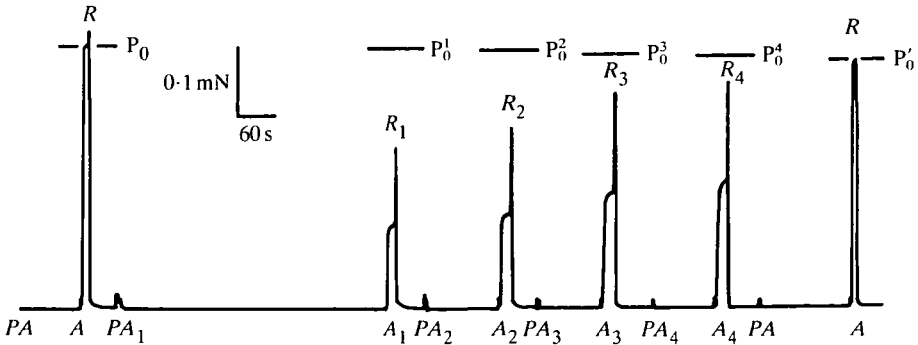
Fig. 1. Example of a series of contraction–relaxation cycles at a low temperature (1·2–1·3 °C) with room temperature (19·0–19·2 °C) control activations before (P_0) and after (P_0') the series. The interpolated values for the control force corresponding to each of the four activations in the series are indicated above each contracture (P_0^{1-4}). The pH (±0·02) of the pre-relaxing (PA), activating (A) and relaxing (R) solutions at room temperature was 7·16 and for the four sets of solutions, PA_1, A_1, R_1; PA_2, A_2, R_2; PA_3, A_3, R_3; PA_4, A_4, R_4, was 6·91, 7·12, 7·34 and 7·52, respectively, at 1·2–1·3 °C. The total concentration of the TES buffer was 150, 140, 85 and 60 mmol l^{-1} in the four sets of solutions (1–4), respectively. The solution set with 150 mmol l^{-1} TES was only occasionally used in this study and contained approximately 6 mmol l^{-1} less K$^+$ than the other sets of solutions. The fibre was obtained from a cane toad acclimated at 16 °C for 6 weeks. Dimensions of the fibre: length, 1·75 mm; diameter, 40 μm; average sarcomere length, 2·22 μm.

temperature. A typical experimental run showing the procedure used to correct for active force deterioration during an experiment is shown in Fig. 1. The procedure includes measuring the maximum Ca^{2+}-activated response at room temperature prior to (P_0) and following (P_0') a number of activations (n) at an experimental temperature. The decline in the maximum force response was divided among the intervening number of contractions, so that the corrected control force value at room temperature (P_0^i) corresponding to a particular contraction in the series (the ith measurement) is given as:

$$P_0^i = P_0 - i(P_0 - P_0')/(n + 1). \tag{1}$$

This interpolation method is similar to that used by Julian (1971). Commonly, a single preparation was activated up to 20 times, incorporating a series of contraction–relaxation cycles at 3–5 temperatures. After several activations at 30 °C and above, the preparations developed a large amount of residual force and the results could no longer be reliably used thereafter (see Results). Fibres were discarded in any experiment when the control, room temperature, response declined by more than 40 % of the initial value.

The room temperature changed by less than 1 °C during an experiment, but differed by up to 6 °C between experiments. Therefore, for purposes of accurate comparisons between different experiments, we activated all preparations at 25 ± 1·0 °C and then finally expressed all the results relative to the equivalent activation response at 25 °C and pH 7·10 in the solution A with 60 mmol l^{-1} TES.

RESULTS

A typical set of results obtained with one fibre preparation from a freshly received toad is shown in Fig. 2. The points are linked by straight lines to allow interpolation of results at different pH values. We have also attempted to measure the pH of freshly dissected skeletal muscle fibres of the cane toad at different temperatures, after rapid maceration with an improvised mortar and pestle, to give a rough estimate of the physiological pH in the cane toad fibres. The iliofibularis, triceps femoris and semimembranosus muscles were carefully dissected and thoroughly blotted on filter paper to remove extracellular fluid. After maceration, the pH was measured continuously for several minutes to allow extrapolation of the pH to time 0. The average value for the extrapolated pH at time 0 obtained at room temperature (23–25 °C) with nine muscles from four toads (±S.E.M.) was $7·20 \pm 0·06$. The average pH value obtained by us falls within the range of the observations made by Malan *et al.* (1976) on *Rana catesbeiana*, but is higher than their average value of 6·90 at 24 °C, possibly because of contamination of our macerated muscle with some plasma, which has a higher pH than the sarcoplasm (Malan *et al.* 1976). Using contralateral muscles placed on ice and measuring the pH of the macerated muscle at 3–7 °C, we obtained an average temperature coefficient (±S.E.M.) of $-0·0175 \pm 0·0085$ pH units °C^{-1} with four paired muscles. This value is very similar to the value
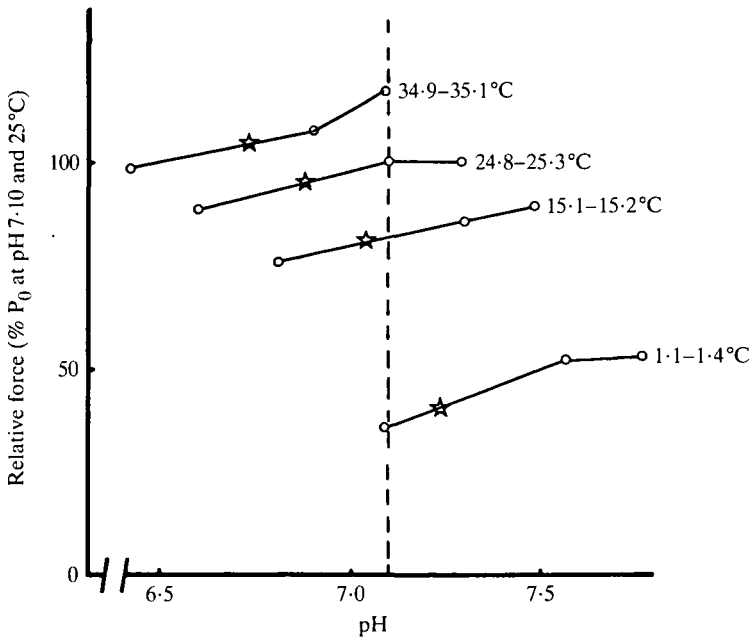

Fig. 2. The relationship between force and pH at four narrow intervals of temperature. Open circles represent the responses from a skinned fibre (length, 1·62 mm; diameter, 50 μm; average sarcomere length, 2·22 μm) prepared from a freshly delivered toad. The vertical dashed line corresponds to pH 7·10. The star symbols represent the values predicted for the *in vivo* sarcoplasmic pH at the corresponding temperature (see text). The data are normalized to the maximal Ca^{2+}-activated response at pH 7·10 and 25 °C.
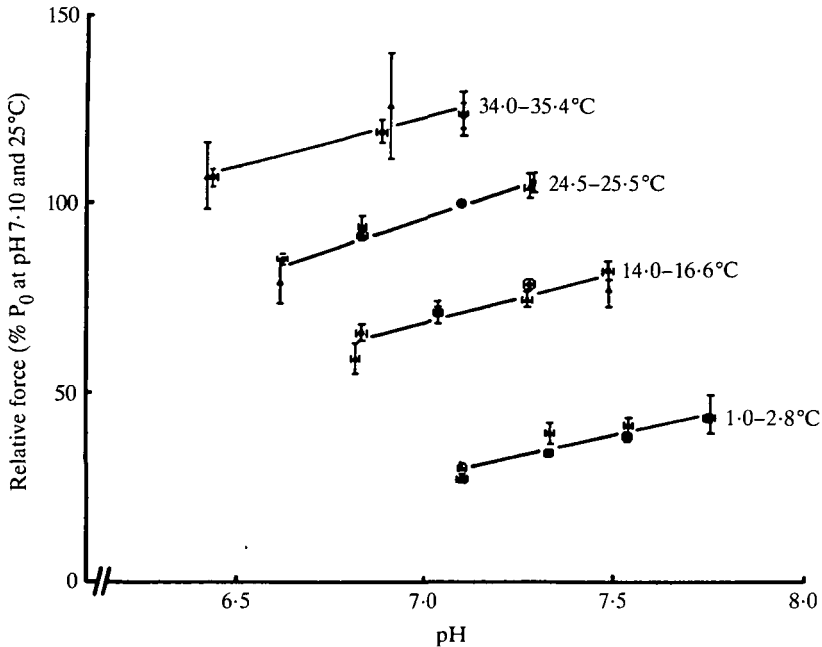
Fig. 3. The relationship between force and pH at four narrow temperature intervals from skinned muscle fibres prepared from 11 toads acclimated at 16°C for 2–12 weeks (●) and from five toads acclimated at 32°C for 11 days to 7 weeks (▲). The data points represent the average results ±S.E.M., when the number of observations indicated below was greater than 3, and the average together with the range of results when the number of observations was equal to or smaller than 3. All results for each fibre were normalized to the maximum Ca^{2+}-activated response at pH 7·10 and 25°C (◓). The solid lines were generated by non-weighted linear regression analysis of the pooled average data points. The correlation coefficients for all the lines were higher than 0·93. Number of observations averaged in order of increasing pH at 1–2·8°C: 19, 6, 14, 10 ● and 10, 3, 7, 3 ▲; at 14·0–16·6°C: 10, 3, 12, 10 ● and 5, 3, 8, 3 ▲; at 24·5–25·5°C: 12, 7, 19, 11 ● and 2, 8, 10, 4 ▲ and at 34·0–35·4°C: 7, 7, 7 ● and 2, 2, 5 ▲ for preparations from cool-acclimated (●) and warm-adapted (▲) animals, respectively.

of $-0·0152$ pH units°C^{-1} reported by Malan *et al.* (1976). Since our results with *Bufo marinus* appear to fall within the range of the more accurate observations made by Malan *et al.* (1976) on *Rana catesbeiana*, we have used their results to estimate the physiological pH for our conditions of temperature. The relative Ca^{2+}-activated force at a given temperature and at physiological pH can then be readily estimated for each fibre by interpolation as shown in Fig. 2 by the star symbols.

Plots of the relationship between relative force and pH in muscles fibres from cool-acclimated and warm-acclimated animals show no statistically significant differences between the two groups of animals (Fig. 3). If the forces measured at one temperature are expressed as a percentage of the maximum force at that temperature then the effect of pH on force will be more pronounced at low temperatures because of the lower absolute force levels at lower temperatures.

An important point which required further investigation was to rule out the possibility that the zwitterionic form of the pH buffer (TES$^{\pm}$) would contribute to

Table 1. *The effects of the zwitterion $TES^{\pm}$ and ionic concentrations on maximum isometric force at room temperature (22–25 °C)*

Solution	$[TES]_{total}$ $(mmol \, l^{-1})$	$[TES^{\pm}]$ $(mmol \, l^{-1})$	$[TES^-]+$ $[propionate^-]$ $(mmol \, l^{-1})$	Relative force* $(\% \pm$ S.E.M.$)$
140 mmol l^{-1} TES	140	100	40	$93 \cdot 7 \pm 2 \cdot 6^a$
60 mmol l^{-1} TES +23 mmol l^{-1} propionate	60	43	40	$96 \cdot 0 \pm 2 \cdot 1^b$
60 mmol l^{-1} TES +80 mmol l^{-1} propionate	60	43	97	$74 \cdot 3 \pm 1 \cdot 9^c$

* The observations ($N = 3$) were made with preparations from a 12-week cool-acclimated toad, and the results were normalized to the maximum force response in the standard solutions with 60 mmol l^{-1} TES (pH 7·10 at 25 °C).
a,b not statistically different ($P > 0 \cdot 2$); *a,c*; *b,c* statistically different at $P < 0 \cdot 001$ (*t*-test).

the ionic strength of the solutions (Miller & Smith, 1984) and thus affect the magnitude of the force response (Fink, Stephenson & Williams, 1986). For this purpose, we performed an experiment in which skinned fibres were maximally activated by Ca^{2+} at room temperature in three sets of solutions: one containing 140 mmol l^{-1} total TES, one other set of solutions where 140 mmol l^{-1} TES was replaced with 60 mmol l^{-1} total TES plus 23 mmol l^{-1} propionate, and another set of solutions containing 60 mmol l^{-1} total TES plus 80 mmol l^{-1} propionate. The solutions were balanced for all other ionic species with our main solutions, with the exception of K^+, which was added as KOH to bring the pH in each solution to $7 \cdot 11 \pm 0 \cdot 02$ at 25 °C. Under these conditions 28·5 % of the TES in solution existed as TES^- and the rest as $TES^{\pm}$, while the propionate was almost fully dissociated. If the zwitterion $TES^{\pm}$ contributed to the ionic strength of the solutions (Miller & Smith, 1984), then the 140 mmol l^{-1} TES solution would have had an ionic strength identical to that of the solution containing 60 mmol l^{-1} TES and 80 mmol l^{-1} propionate. However, if the zwitterion $TES^{\pm}$ did not contribute to the ionic strength, then the 140 mmol l^{-1} TES solution would have had a similar ionic strength to that of the solution containing 60 mmol l^{-1} TES and 23 mmol l^{-1} propionate. The results presented in Table 1 clearly show that the maximum Ca^{2+}-activated force response was almost the same in the solutions with 140 mmol l^{-1} TES and 60 mmol l^{-1} TES plus 23 mmol l^{-1} propionate, indicating that $TES^{\pm}$ does not affect the ionic strength of the solutions as perceived by the contractile apparatus.

The relationships between experimental temperature and maximum Ca^{2+}-activated force in skinned muscle fibres at constant pH ($7 \cdot 10 \pm 0 \cdot 05$) from cool- and warm-acclimated toads are shown in Fig. 4. The maximum Ca^{2+}-activated force increased about four- to five-fold when the temperature was increased from about 2° to 35 °C, regardless of the temperature at which the animals were acclimated. The results were similar for both groups of toads and were not statistically different (*t*-test, $P > 0 \cdot 10$) from each other at any of the temperatures investigated. If allowance were made for the change in the physiological pH with temperature, then the dependence between maximum Ca^{2+}-activated force and temperature would

become less marked for both groups of animals (Fig. 5), because of the compensatory effect of increased pH at lower temperatures. On average, the maximum Ca^{2+}-activated force increased by a factor of 3·3 and 3·2 for the cool- and warm-acclimated groups of animals, respectively, when the temperature increased from approximately 2° to 30°C. Again, no statistically significant differences could be noticed between the preparations from cool- and warm-acclimated animals at any temperature and after any period of acclimation up to 80 days, indicating that short-term acclimation does not modify this property of the contractile apparatus.

In absolute terms, the average (±S.E.M.) maximum Ca^{2+}-activated tension developed in the skinned muscle fibres at room temperature (18–24°C) under our conditions was $408 \pm 46\,kN\,m^{-2}$ ($N = 24$) and $404 \pm 45\,kN\,m^{-2}$ ($N = 26$) for preparations dissected from cool- and warm-acclimated animals, respectively. The tension values were calculated from the first or second maximal Ca^{2+}-activated response and from the apparent diameter of the preparation, measured under oil, and the cross-section of the preparations was assumed to be circular. No statistically significant difference was found between the results obtained with the two groups of animals.
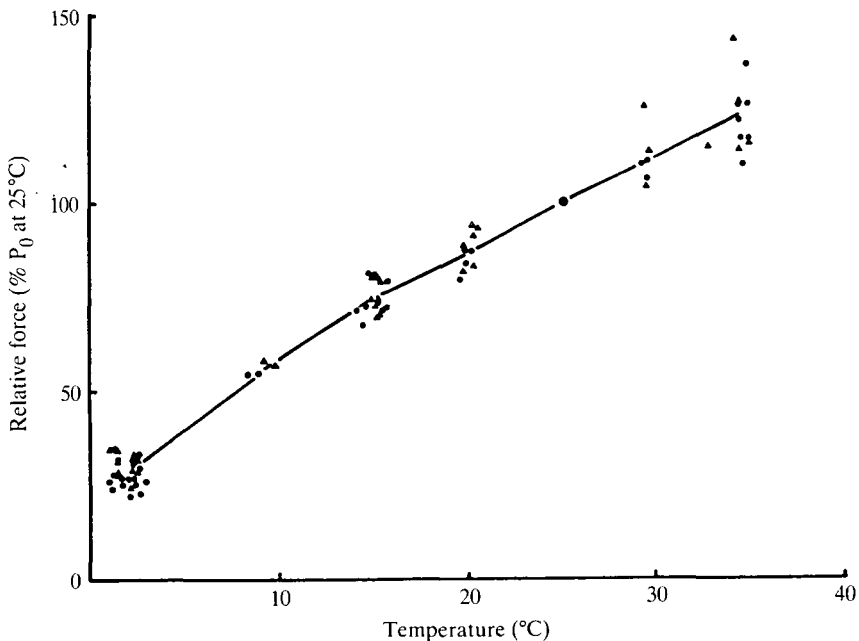

Fig. 4. The relationship between experimental temperature and maximum Ca^{2+}-activated isometric force in skinned muscle fibres from cool-acclimated (●) and warm-acclimated animals (▲) at pH 7·10. Each symbol represents an individual response from individual preparations and the lines were drawn through the mean force value of all results at each narrow interval of temperature. All data are expressed as percentages of the response of each individual fibre at $25 \pm 0.5\,°C$. The results were obtained with 19 fibres from 11 cool-acclimated and 12 fibres from five warm-acclimated toads (see also Fig. 3).
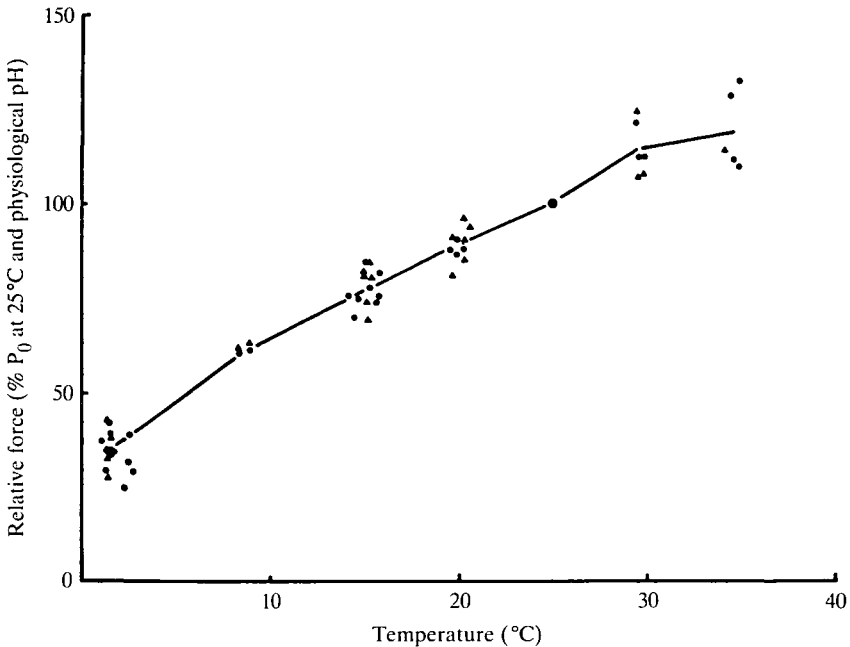
Fig. 5. The relationship between experimental temperature and the maximum Ca^{2+}-activated force at predicted physiological pH (see text) in skinned muscle fibres from cool-acclimated (●) and warm-acclimated (▲) animals. Each symbol represents the interpolated relative force value at given temperature from an individual fibre (see Fig. 2). All results were normalized to the response at 25°C. The results were obtained with 12 and nine fibres for ● and ▲, respectively, using the same animals as for Fig. 3.

In another series of experiments, we used the pH buffer PIPES, which did not require substantial changes in its total concentration (within $2\,\mathrm{mmol\,l^{-1}}$) to maintain a constant ionic strength in the solutions at different temperatures. In these experiments creatine phosphokinase ($15\,\mathrm{units\,ml^{-1}}$) was present in all solutions. We also estimated the stiffness in the preparations from the amplitude of the force oscillations generated by the sinusoidal length oscillations (<0·6% fibre length) at 2·5 kHz. The force and stiffness results obtained at pH 7·10 ± 0·02 with 3- to 8-week cool-acclimated animals are plotted against temperature in Fig. 6. The force–temperature relationship is essentially the same as that shown in Fig. 4 when the pH buffer TES was used with no creatine phosphokinase added to the solutions. The stiffness in the preparations appears to decrease significantly below about 20°C and to remain unchanged above 25°C. The same trend in the stiffness results was obtained with two preparations from a cool-acclimated (8 weeks) and three preparations from a warm-acclimated (8 weeks) toad when applying sinusoidal length oscillations (<1%) at 0·5 kHz in the standard solutions containing the TES pH buffer. In these experiments, the average values (±S.E.M.) for the relative stiffness at 2–3°C, 14·5–15·5°C and 33·5–35°C compared with that at 25°C were 51·1 ± 3·4%, 88·0 ± 2·1% and 102·7 ± 0·7% for the fibres from the warm-acclimated animal and 57 ± 6%, 90 ± 1% and 101 ± 4% for the fibres from the

cool-acclimated animal. Qualitatively, the apparent stiffness in the skinned fibres at 0·5 kHz was lower than that at 2·5 kHz, as previously reported by Cecchi, Griffiths & Taylor (1982), but the relative stiffness in maximally Ca^{2+}-activated fibres from both cool- and warm-acclimated animals followed the same relationship with temperature as that shown in Fig. 6.

The maximum force–temperature relationship appears to be influenced by the general condition of the animals. Force responses obtained at low temperatures in fibres from the first batch of warm-acclimated toads (fed 4 crickets animal^{-1} week^{-1}) were noticeably lower than responses elicited from individuals in subsequent batches (fed 8–10 crickets animal^{-1} week^{-1}). The results at 1–5°C were statistically different ($P < 0·05$, t-test) between the first group and the other groups of warm-acclimated toads, suggesting that food availability can affect the temperature dependence of maximum Ca^{2+}-activated force response more readily than temperature acclimation. However, no difference was noticed between fibres dissected from cool-acclimated animals (4–8 weeks) which had food available to them or were not fed at all.

A complicating problem with the maximal Ca^{2+}-activation at temperatures higher than 15°C was the inability to 'relax' the preparations fully after prolonged activation, even when [Ca^{2+}] was decreased to below 10^{-9} mol l^{-1} (see inset in

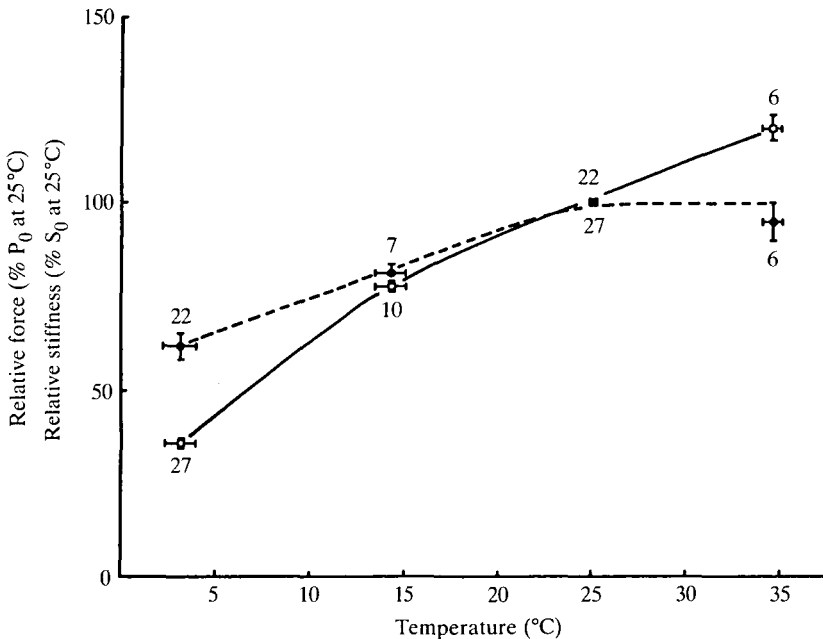Fig. 6. The effect of temperature on maximum Ca^{2+}-activated force (□) and stiffness (●) at pH 7·10. The results were normalized to the maximum force (P_0) and stiffness (S_0) values at 25°C and were obtained with eight preparations from 3- to 8-week cool-acclimated toads (three animals). The number of observations at each temperature range, indicated by the horizontal bars, is shown by each point. The vertical lines indicate ±S.E.M. The curves were drawn by eye.

Fig. 7A). The amount of 'irreducible force' or 'residual resting force' developed in our preparations was directly related to the temperature of the solutions and to the duration of activation. Residual force did not occur spontaneously upon incubation at high temperature if the fibres were not activated. Fig. 7A illustrates the increment in the residual force as a function of the duration of activation at different temperatures for the cool-acclimated animals. The results can be well fitted by straight lines at all temperatures investigated (Fig. 7A). The average increment of residual force per unit time increases with power 2·66 of the temperature at which contraction was elicited (Fig. 7B). Average results obtained with preparations from warm-acclimated toads are also included in Fig. 7B and are fitted by a different curve which clearly diverges from the curve for cool-acclimated animals as the temperature drops below 25°C. A statistical analysis of the results (t-test) indicates that the data points ($\pm$S.E.M.) at 15°C, $0·050 \pm 0·018\,\%P_0$ at $25°C\,s^{-1}$, $N = 8$, for cool-acclimated and $0·02 \pm 0·02\,\%P_0$ at $25°C\,s^{-1}$, $N = 6$, for warm-acclimated animals are statistically different ($P < 0·05$). The results obtained at temperatures higher than 20°C were not statistically different ($P > 0·10$) between the cool- and warm-acclimated toads.

Stiffness measurements of relaxed fibres displaying significant residual force have indicated that stiffness in the fibres was approximately linearly related to the residual force. The transfer of the fibres from a relaxing solution at a given temperature to another relaxing solution at a different temperature did not result in a rapid modification of the residual force or of the associated stiffness. The amount of residual force developed in the preparations was not modified by the presence of $10\,mmol\,l^{-1}$ of the reducing agent dithiothreitol (Jewell & Kentish, 1981), or by the presence of creatine phosphokinase ($15\,units\,ml^{-1}$) in solutions. Residual force redeveloped only slowly and to a much lower level following a rapid partial release of the skinned fibres. These observations are consistent with the idea that residual force in the relaxing solution may be due to cross-bridges which are cycling at a very slow rate compared with those in the fully Ca^{2+}-activated preparations.

DISCUSSION

The thermal dependence of maximum force production in Ca^{2+}-activated skinned muscle fibres from the toad *Bufo marinus* contrasts with the relative thermal independence of maximum force production exhibited by other anurans. The maximum force in skinned of *B. marinus* dropped to about 30% of the 25°C response at 1–2°C, when the pH in solutions was maintained at 7·10, and to about 40%, if the pH in solutions followed the physiological trend in anuran muscle (Malan *et al.* 1976). Maximum tetanic tension in the intact iliofibularis muscle of *B. marinus* also showed great sensitivity to reduction in temperature, dropping to 10–20% of the room temperature value when the temperature was lowered to 3–4°C (B. B. Rees & D. G. Stephenson, unpublished observations). Gibbs & Chapman (1974) also reported that the tetanic force of isolated sartorius muscles of *Bufo marinus* increased by more than 20% when the temperature was raised from 10 to

20°C and that they were not able to stimulate the muscle at 0°C. In contrast to these results, Bressler's (1981) study on *Bufo bufo* showed that maximum tetanic force in sartorius muscles at 0°C was 73% of the response obtained at 20°C, and Renaud & Stevens (1981b) found with another toad, *Bufo americanus*, that the sartorius muscle could generate 77% of the tetanic tension at 5°C compared with the response at 25°C. Published results with intact frog muscles (*Rana* species) are similar to the results on *B. bufo* and *B. americanus* (see Bennett, 1984, for a review), and studies with skinned frog muscle fibres (Godt & Lindley, 1982; Moisescu & Thieleczek, 1978) have further confirmed the relative thermal independence of force production in anuran muscle.

The difference between skeletal muscles of *Bufo marinus* and those of other anurans with respect to their abilities to generate force at low temperature is striking
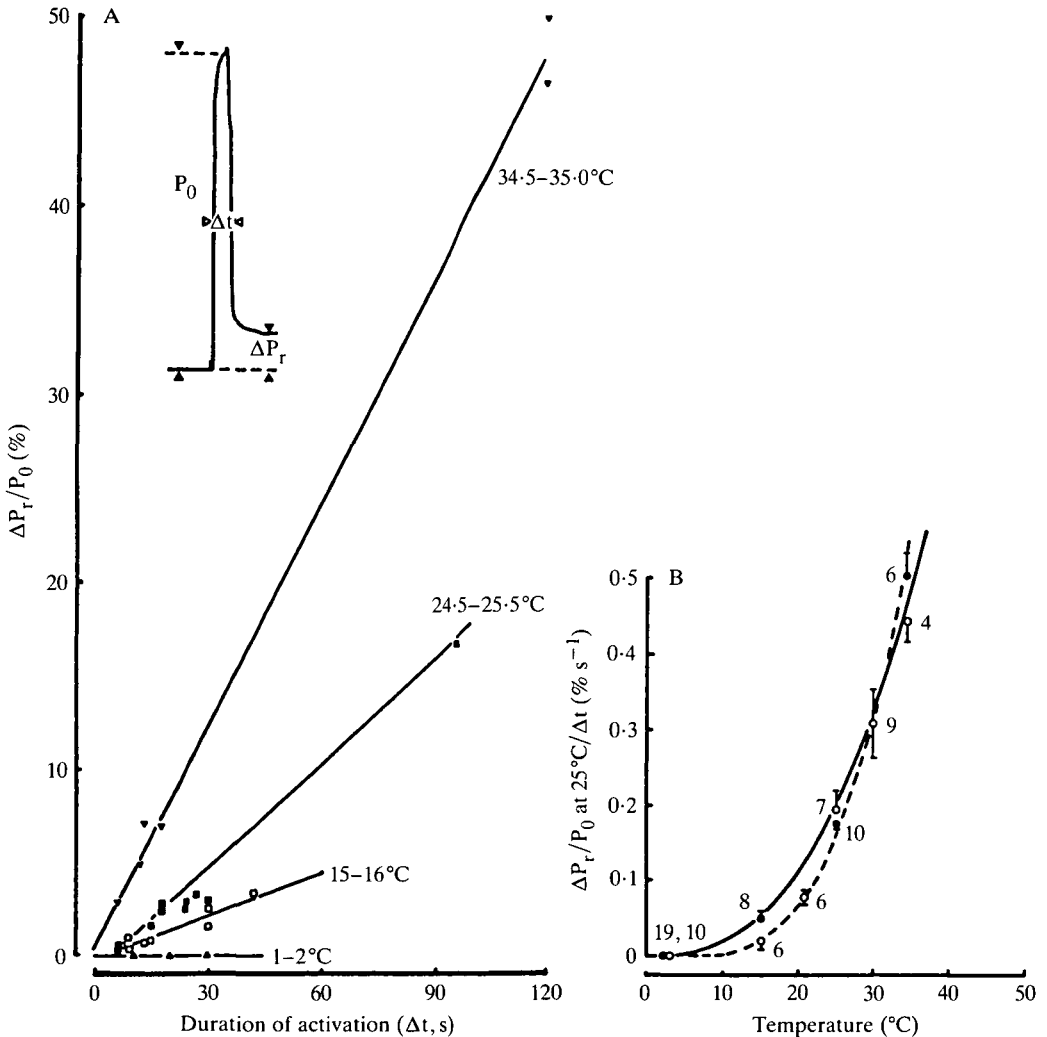

Fig. 7

and requires consideration of the mechanism responsible for this difference to occur. Clearly the mechanism must operate at the level of the contractile apparatus, since these differences are apparent not only in the intact fibres (Gibbs & Chapman, 1974; B. B. Rees & D. G. Stephenson, unpublished observations) but also in the skinned muscle preparations when the excitation process was bypassed by the direct activation of contraction by Ca^{2+} under controlled conditions. Then, in the sliding-filament model of muscle contraction (Huxley, 1974), the decline in maximum force at lower temperatures can be the result of fewer active cross-bridges, a reduction in the force generated per cross-bridge or a combination of both of these factors. Studies on other anurans exhibiting a relative thermal independence of maximum force production have shown that stiffness in the muscle fibres either remained virtually constant as the temperature was lowered from 20 to 0°C while maximum tetanic force dropped by 27% (*Bufo bufo*, Bressler, 1981), or that it changed less than the maximum tetanic force when the temperature was varied between 0 and 8°C (*Rana temporaria*, Ford, Huxley & Simmons, 1977). Since relative stiffness measurements can be used in a first approximation as an index of the relative number of cross-bridges in the preparation (Huxley, 1974; Cecchi *et al.* 1982; Schoenberg & Wells, 1984), these results were interpreted to suggest that the number of cross-bridges in these anuran muscle fibres did not decline with temperature and that the amount of force per cross-bridge decreased slightly with temperature.

In our study, the stiffness of the maximally Ca^{2+}-activated skinned fibres of *Bufo marinus* at both 0·5 and 2·5 kHz increased by a factor of about 1·6 between 3–4°C and 25°C and reached a plateau at temperatures higher than 25°C (Fig. 6). This result is unlike that from other anurans and suggests that the number of attached cross-bridges during maximum Ca^{2+}-activation may decline with temperature in the skeletal muscle fibres of *Bufo marinus*. However, the apparent reduction in the number of cross-bridges at low temperatures cannot fully explain the more marked

Fig. 7. (A) The dependence of the increment of residual force in fibres from cool-acclimated toads after maximal activation at certain temperatures. The increment of residual force is expressed as the ratio between the net increase in residual force, ΔP_r, (see inset) and the maximum active force, P_0, at that temperature. The duration of the activation, Δt, was measured at half peak (P_0) height as illustrated in the inset. Changes in P_r following contractions of up to 30 s at 1–2°C were below the resolution of the apparatus and were assigned a value of zero. The lines were obtained by linear regression analysis and the correlation coefficients support the rectilinear nature of all relationships at $P < 0·001$. (B) Temperature dependence of the average increments of residual force in fibres from cool-acclimated (●) and warm-acclimated (○) toads after 1 s maximal Ca^{2+}-activation at the respective temperature. The results are expressed as percentage of the maximum force at 25°C (P_0^{25}) per unit time. The average data points were fitted by regression analysis to equations of the type $y = ax^b$. The value of a ($\%P_0^{25} s^{-1} °C^{-1}$) was $3·8 \times 10^{-5}$ for ● and 8×10^{-7} for ○, and b was 2·66 for ● and 3·79 for ○. The correlation coefficients were 0·995 for both curves. The number of observations is indicated next to each average point ±S.E.M. The results for cool-acclimation in A and B were obtained with eight preparations from six cool-acclimated toads, and the results for warm-acclimation were obtained with eight preparations from four warm-acclimated toads. The results for 15°C are significantly different (*t*-test, $P < 0·05$) between the two groups of animals.

temperature effect on the maximum force. Therefore, the most likely explanation of our results is that the decline in force at lower temperatures is due to both a reduction in the number of cross-bridges between the myosin and actin filaments and to a decrease of force generated per cross-bridge, similar to that observed in other anurans.

There is no evidence to suggest that a different isomyosin pattern of the *Bufo marinus* fast muscles may be responsible for the different temperature dependence of maximum Ca^{2+}-activated force, because according to J. F. Y. Hoh (quoted by Lännergren & Hoh, 1984) the myosin extracted from predominantly fast muscles of the cane toad yielded a similar three-band isomyosin pattern to that characteristic of predominantly fast muscles of temperate anurans (Pliszka, Strzelecka, Pantaloni & d'Albis, 1981).

Whatever the precise mechanism responsible for the marked temperature dependence of maximum force in *Bufo marinus* compared with other anurans, one can understand better the interspecific differences between various anurans when the thermal environments of the individual species are considered. Thus, the majority of the research on muscle contraction in anurans has been carried out with species from temperate habitats in which ambient temperatures may change by more than 10 °C in one day and by more than 30 °C annually. A broad range of thermal independence of muscular ability would allow a temperate anuran to maintain a high degree of activity in such an environment. Brattstrom (1963) cites voluntary minimum and maximum body temperatures of *Bufo americanus* as 11·2 and 32·3 °C, respectively, for this temperate species of toad. These temperatures correspond to the lowest and highest body temperature measurements made on toads found to be active in the field. Over a 20° temperature range (5–25 °C), which is close to the range of body temperature in this animal, the maximum force response in intact sartorius muscle increased by a factor of 1·19 when physiological pH was maintained in the bathing solutions (Renaud & Stevens, 1981*b*). *Bufo marinus*, however, is tropical in distribution and not subjected to great diurnal or annual temperature fluctuations. Twenty-five measurements of body temperature of the cane toad in the field varied over only 5 °C between 22·0 and 27·0 °C (Brattstrom, 1963). Interestingly, the maximum force response in skinned fibres at physiological pH over this restricted range of temperature increased also by a factor of about 1·18 (Fig. 4).

If the thermal environment appears to be so important in determining the temperature dependence of maximum force in these two species of toad, it was particularly interesting to discover that acclimation of *B. marinus* to different thermal regimes for up to 80 days modified neither the maximum isometric force, nor the temperature and pH dependence of the isometric force. These findings are in general agreement with recent results from acclimation studies of temperate anurans (Renaud & Stevens, 1981*a,b*; Rome, 1982). The inability of the cane toads to sustain high tensions in their muscles at low temperatures and to compensate for this temperature dependence by short-term thermal acclimation may be the major factors which limit the distribution of the cane toads.

The only significant effect of acclimation found in this study was the decrease of the rate of residual force development at temperatures lower than 20 °C following acclimation at higher temperatures. Although it is difficult to ascribe physiological importance to this result, this finding can nevertheless have profound implications for muscle physiology, because it may provide a way to minimize the residual force in anuran skinned fibres, which can grossly interfere with a number of critical mechanical measurements (Julian *et al.* 1986). The results on residual force obtained in this study indicate that the fractional increase in residual force developed in the skinned fibres is linearly related to the duration of activation at any given temperature. Furthermore, for the same duration of activation, the amount of residual force developed is very sensitive to the temperature at which activation is elicited. The results emphasize the importance of producing a fast activation of the skinned fibre preparations (see Materials and Methods) in order to minimize this development of residual force. These results complement the study of Thames *et al.* (1974) on frog residual force and are also related to the recent findings of Johnston & Altringham (1985) on skinned fish muscle fibres which show that at a given temperature the magnitude of residual force is related to the normal thermal environment of the respective animal. The residual force was always smaller in the fishes living in warmer environments. Without further experimentation it is not possible to explain the exact mechanism responsible for the formation of cross-bridges in the absence of Ca^{2+} and in the presence of high concentrations of MgATP, but one cannot exclude the possibility, suggested by Johnston & Altringham (1985), that this phenomenon may be due to the partial loss of Ca^{2+} sensitivity of the troponin–tropomyosin regulatory system. Since the kinetics of formation of these cross-bridges is much slower than the kinetics of formation of the cross-bridges in the fully Ca^{2+}-activated fibres (Thames *et al.* 1974; see also Results), the explanation of Johnston & Altringham (1985) would also imply that Ca^{2+} can modify the kinetics of formation of cross-bridges, for which there is strong recent evidence (Julian *et al.* 1986). Alternatively, the cross-bridge formation responsible for residual force could be connected to the mechanism of cross-bridge formation in the resting fibres that have been suggested by Hill (1968) and Chalovich & Eisenberg (1982).

The results from this study also allow a better understanding of two more properties of skeletal muscle. First, evidence is provided (Table 1; Fig. 6) that the contractile apparatus is not sensitive to the concentration of the zwitterionic species in solutions and therefore, as a first approximation, it is not necessary to take these species into consideration when estimating the ionic strength of the solutions (Miller & Smith, 1984; Fink *et al.* 1986). Second, the results on the pH effect on maximum Ca^{2+}-activated force show that temperature can be an important factor in determining the dependence of isometric force on pH. At room temperature our results are similar to those of Fabiato & Fabiato (1978). If the change in force is normalized to the response obtained at a certain pH and temperature, then the pH appears to have a much stronger influence on the isometric force at lower temperatures. This effect can be of physiological importance because as the temperature drops, the pH in

the sarcoplasm is expected to rise (Malan *et al.* 1976) and this would compensate in part for the loss of force at reduced temperatures (see Fig. 5).

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