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PARVALBUMIN CONTENT AND Ca^{2+} AND Mg^{2+} DISSOCIATION RATES CORRELATED WITH CHANGES IN RELAXATION RATE OF FROG MUSCLE FIBRES

BY TIEN-TZU HOU, J. DAVID JOHNSON* AND JACK A. RALL

*From the Department of Physiology and *the Department of Physiological Chemistry, Ohio State University, Columbus, OH 43210, USA*

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SUMMARY

1. Experiments were done to test the hypothesis that parvalbumin (PA) promotes relaxation in frog skeletal muscle. Single fibres and purified PA from *Rana temporaria* skeletal muscle were used to determine the relationship between PA concentration ([PA]), Ca^{2+} and Mg^{2+} dissociation rates from PA and changes in rate of relaxation as a function of tetanus duration at 0 °C.

2. Total [PA] in fibres from tibialis anterior muscles is $0{\cdot}76 \pm 0{\cdot}20$ mmol PA l^{-1} myoplasmic water (mean ± S.D., $n = 25$) with 65% PA IVa and 35% PA IVb, where PA IVa and PA IVb are PA isoforms.

3. Relaxation rate from an isometric tetanus slows progressively as a function of tetanus duration with an exponential time course and a rate constant of $1{\cdot}18 \pm 0{\cdot}35$ s^{-1} ($n = 17$). Time course of recovery of relaxation rate after a prolonged tetanus is exponential with a rate constant of $0{\cdot}12 \pm 0{\cdot}02$ s^{-1} ($n = 14$).

4. The extent of recovery of relaxation rate after a prolonged tetanus was correlated with total [PA] in fibres (correlation coefficient (r) = 0·80, $n = 7$; $P < 0{\cdot}05$).

5. Dissociation rate constants for Mg^{2+} and Ca^{2+} from purified PA are $0{\cdot}93 \pm 0{\cdot}02$ s^{-1} ($n = 5$) and $0{\cdot}19 \pm 0{\cdot}01$ s^{-1} ($n = 5$), respectively. Dissociation rate constants were not significantly different for PA isoforms IVa and IVb. These rate constants are similar to the rate constants determined for the time courses of slowing and recovery of relaxation rate, respectively.

6. Results suggest that the time courses of slowing and recovery of relaxation rate may be controlled, to a large extent, by Mg^{2+} and Ca^{2+} dissociation from PA, respectively. This evidence supports a role for PA in facilitating relaxation during a tetanus in frog skeletal muscle at 0 °C.

INTRODUCTION

Parvalbumin (PA) is a soluble Ca^{2+}-binding protein found in high concentration in the sarcoplasm of vertebrate fast-contracting skeletal muscle (for review see Heizmann, 1984). PA binds two Ca^{2+} per mole with high affinity (−log of dissociation constant (pK_d) of 8) in competition with Mg^{2+} (pK_d of 4) (Potter, Johnson, Dedman, Schreiber, Mandel, Jackson & Means, 1977). It has been hypothesized that PA

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promotes relaxation in fast-contracting skeletal muscle (Briggs, 1975; Gerday & Gillis, 1976; Pechere, Derancourt & Haiech, 1977). Under resting conditions, PA is thought to be loaded predominantly with Mg^{2+} (Haiech, Derancourt, Pechere & Demaille, 1979). The net rate of Ca^{2+} uptake by PA during contraction would be determined by the rate of dissociation of Mg^{2+} from PA. This rate is much slower than the rate of Ca^{2+} binding to Ca^{2+}-specific sites of troponin (TN) (Robertson, Johnson & Potter, 1981). Thus Ca^{2+} would preferentially bind to TN during muscle activation. In contrast, PA can deplete isolated myofibrils of Ca^{2+} and isolated sarcoplasmic reticulum (SR) can deplete PA of Ca^{2+} (Gerday & Gillis, 1976). Simulation studies indicate that, in theory, Ca^{2+} exchange for Mg^{2+} on PA can occur rapidly enough for PA to promote relaxation in frog skeletal muscle by acting in parallel with the SR (Gillis, Thomason, Lefevre & Kretsinger, 1982; Baylor, Chandler & Marshall, 1983; Cannell & Allen, 1984). Furthermore, the level of PA in skeletal muscle correlates positively with relaxation speed (Heizmann, Berchtold & Rowlerson, 1982).

If PA promotes relaxation, its effect would be expected to diminish as tetanus duration increases since PA would progressively fill with Ca^{2+}. Since PA is freely soluble in the myoplasm (Gillis, Piront & Gosselin-Rey, 1979), relaxation rate should decrease with increased tetanus duration with a time course determined by the rate of Mg^{2+} dissociation from purified PA. Relaxation rate does slow progressively with increased tetanus duration in frog whole muscles (Abbott, 1951; Curtin, 1986; Peckham & Woledge, 1986) and fibres (Blinks, Rudel & Taylor, 1978). Furthermore the time course of recovery of relaxation rate after a prolonged tetanus would be predicted to be limited by the rate of Ca^{2+} dissociation from PA. Recovery of relaxation rate should be slower than that for progressive slowing of relaxation rate because Ca^{2+} dissociates from PA more slowly than does Mg^{2+} (Potter, Johnson & Mandel, 1978; Ogawa & Tanokura, 1986*b*). The observed time course of recovery of relaxation rate in frog whole muscles is slower than the time course of progressive slowing of relaxation rate (Curtin, 1986; Peckham & Woledge, 1986).

To test quantitatively the hypothesis that PA promotes muscle relaxation, the following relationships were determined using frog muscle fibres and purified frog PA: (a) time course of slowing of relaxation rate with increasing tetanus duration compared to rate of Mg^{2+} dissociation from PA, (b) time course of speeding up of relaxation rate after a prolonged tetanus, with increasing rest interval, compared to rate of Ca^{2+} dissociation from PA and (c) magnitude of changes in relaxation rate compared to PA concentration ([PA]).

Preliminary reports of some of these results have been published previously (Hou, D'Anniballe & Rall, 1988, 1989; Hou, Johnson & Rall, 1989, 1990).

METHODS

Fibre preparation and experimental set-up

Frogs, *Rana temporaria*, were killed by decapitation and the spinal cord destroyed. Then single fibres with intact membranes and tendons were isolated from tibialis anterior muscles. Frogs were purchased from Charles Sullivan of Nashville, TN, USA and kept at 5 ± 1 °C. Fibres were bathed in a solution containing (mM): NaCl, 95; $NaHCO_3$, 20; KCl, 2·5; $CaCl_2$, 1·0; $MgCl_2$, 1·0; glucose, 11·1; saturated with 95% O_2–5% CO_2; pH, 7·2. After dissection, small holes were made in tendons as near as possible to the fibre. Fibres were mounted horizontally in an experimental chamber with

one tendon attached to the hook of a force transducer (Cambridge Technology, MA, USA), and the other tendon attached to a hook fixed to the opposite end of the chamber. Temperature was maintained at $0 \pm 0{\cdot}1$ °C by circulating fluid through channels in an aluminium block upon which the chamber was mounted. Resting sarcomere length was determined by He–Ne laser diffraction and set at 2·2 μm. Fibres were stimulated in solution via a pair of platinum plate electrodes which were mounted parallel to the fibres in the walls of the chamber. Rectangular pulse magnitude was set at 1·6 times threshold voltage. Stimulus parameters for a tetanus were 0·2 ms pulse duration and frequency of 10–15 Hz. Isometric tetanus force was recorded on a chart-recorder (Gould) and a digital oscilloscope with minidiskette storage (Nicolet) arranged in parallel.

Experimental protocol for mechanical experiments

Analysis of relaxation rate

Relaxation rate from an isometric tetanus was calculated as the inverse of the time required for force to fall from 95 to 80% ($1/t_{95\text{–}80\%}$) of the peak value after the last stimulus. The 80% point occurred before the 'shoulder' of relaxation. This measure of relaxation rate was used because firstly, it occurs during the phase of relaxation where sarcomere length is constant and thus the fibre is truly isometric (Edman & Flitney, 1982) and secondly, it significantly correlates with changes in rate of fall of the calcium transient during relaxation (Cannell, 1986). For completeness, relaxation rate also was measured as the inverse of time for force to fall by 5% ($1/t_{5\%}$) or by 50% ($1/t_{50\%}$) of peak value after the last stimulus. Also a rate constant was calculated for the exponential component of relaxation (the last 60% of force decline, ξ_{exp}).

Time course of slowing of relaxation rate with increasing tetanus duration

After fibres were rested for 30 min, five to seven isometric tetani of various durations were produced in a random order. These tetani varied from 0·3 to 5 s duration. The shortest tetanus duration was one where peak force was just attained. Occasionally, a tetanus of 10–20 s duration was given. Tetanic contractions were separated by rest intervals of 30 min in order to allow complete metabolic recovery. Relaxation rate as a function of tetanus duration was fitted to an exponential equation. After the experiment, fibre volume was measured and some fibres were analysed for PA concentration.

Recovery of relaxation rate after a prolonged tetanus with increasing rest interval

Fibres were given conditioning (0·5–20 s) and test (0·5 s) tetani in pairs. The duration of the test tetanus was selected to just produce peak force. Rest interval between pairs was 30 min. Five to seven pairs of tetani were given in random order. Rest intervals between conditioning and test tetani varied from 0·8 to 30 s. Relaxation rate of the test tetanus was divided by relaxation rate of the conditioning tetanus of its pair. This procedure corrected for variation in fibre performance throughout an experiment. The average coefficient of variation (S.D./mean) ($n = 14$) for the relaxation rate in the 4 s conditioning tetanus during an experiment was 4·9%. The time course of recovery of this ratio of relaxation rates with increasing rest interval was fitted with an exponential equation. After the experiment, fibre volume was measured and some fibres were analysed for PA concentration.

PA identification and concentration determination in fibres

PA standards

Purified PA was used to identify PA isoforms in fibres and as standards in gels to construct calibration curves. Purified isoforms from *Rana temporaria*, PA IVa (pI (−log of isoelectric point) 4·97) and IVb (pI 4·75) (Gosselin-Rey & Gerday, 1977), were purchased from C. Gerday, University of Liege and stored as lyophilized powder at −70 °C. The amount of PA in solution was determined routinely by weighing the lyophilized powder using an automatic electrobalance (CAHN 29, Cerritos, CA, USA) with an accuracy of 0·1 μg. Subsequently, concentrations of PA were determined from their molar absorption coefficients at 259 nm (IVa, 2934 ± 147 (mean ± S.E.M.) and IVb, 2752 ± 138 (mean ± S.E.M.) $mol^{-1}\,cm^{-1}$) (Gosselin-Rey & Gerday, 1977). The ratio of [PA] determined from absorption coefficient to that determined from weight was $0{\cdot}88 \pm 0{\cdot}08$ ($n = 6$) for IVa and $0{\cdot}74 \pm 0{\cdot}03$ ($n = 9$) for IVb. These ratios were used as correction factors to determine true PA amount and concentration.

One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

PA content in muscle samples was determined by SDS PAGE (Laemmli, 1970). Fibre proteins were denatured by heating at 100 °C for 15 s in sodium dodecyl sulphate (SDS) buffer containing 5% SDS, 6·67% glycerol, 20·83 mM-Tris, 40 mM-dithiothreitol and a trace amount of Bromphenol Blue at pH 6·8. 5 μl of sample buffer was loaded onto a slab gel, which was 0·75 mm thick and contained twenty wells. Concentrations of acrylamide in the stacking and separating gels were 6 and 15% respectively. After loading samples, samples were stacked for 30 min at 15 mA and run for approximately 3·5 h at 19 mA with temperature maintained at 20–23 °C. Gels then were (a) dialysed for approximately 30 min in a fixing solution containing 50% methanol and 30% acetic acid, (b) stained in 1% Coomassie Brilliant Blue R-250, 50% methanol and 10·5% acetic acid for approximately 2·5 h, (c) destained in a 10% methanol and 10% acetic acid solution in a Bio Rad (model 556) destainer for about 24 h, (d) dried between cellophane membranes using a gel dryer (Bio Rad) and (e) scanned on a densitometer (LKB·Ultro Scan XL, Bromma, Sweden).

Two-dimensional gel electrophoresis

In order to verify that each band of PA seen in one-dimensional gels contained only one protein, a two-dimensional PAGE system (O'Farrell, 1975), the Mini-PROTEAN II 2-D Cell (Bio Rad) system, was used. Muscle samples were stored in the first-dimension sample buffer containing 9·5 M-urea, 2·0% Triton X-100, 5% β-mercaptoethanol, 1·6% Bio-lyte 4/6 ampholyte and 0·4% Bio-lyte 3/10 ampholyte. First-dimension tube gels (isoelectric focusing gels) were made up of 9·2 M-urea, 4% acrylamide, 2·0% Triton X-100, 1·6 Bio-lyte 4/6 ampholyte, 0·4% Bio-lyte 3/10 ampholyte, 0·01% ammonium persulphate and 0·1% *N,N,N',N'*-tetramethylethylenediamine (TEMED), which resulted in a linear pH gradient from pH 3 to 10. Samples corresponding to 250 μg muscle (dry wt) were loaded onto tube gels and run at 200 V for 10 min, 300 V for 15 min and then at 400 V for 5·5 h. After the first-dimension run, tube gels were dispersed into a Petri dish and slightly wetted with electrophoresis running buffer and stored at −70 °C until the second-dimension gels were ready. These were SDS polyacrylamide slab gels prepared as described above except that a standard well for the second-dimension run was made for loading the tube gel. After laying the tube gel on the well, 75 μl of SDS sample buffer was added to cover the tube gel completely. The second-dimension gel was stacked at 5 mA for 20 min and run at 8 mA for approximately 3 h. Gels were stained, destained and dried as described above.

Determination of fibre volume

A fibre was stretched to a sarcomere length of 3·5 μm where the cross-section is nearly circular (Blinks, 1965). Width at five to seven points along the fibre was measured at 400 × magnification in order to calculate circular cross-sectional area. Fibre length was also measured. Fibre volume was determined as the product of length and cross-sectional area.

Determination of [PA] in fibres

After a mechanical experiment and measurement of volume, a fibre was cut from its tendons and stored in 5 μl of SDS sample buffer at −70 °C until protein analysis by SDS PAGE. Quantity of PA (in μg) in a fibre was determined by comparison of densitometric scans of fibre PA with linear calibration curves from four to six different amounts of each PA standard in the range 0·9–0·045 μg run on the same gel. [PA] in fibre myoplasmic water was determined as the ratio of PA amount (in μmol) to 0·71 × fibre volume (in l), i.e. 71% of fibre volume is water which exists outside of SR and mitochondria (Baylor *et al.* 1983). Myoplasmic water is the volume of interest since PA is localized to the space outside of SR and mitochondria.

Determination of steady-state and kinetic properties of Ca^{2+} and Mg^{2+} interaction with PA

Steady-state luminescence experiments

Terbium (Tb^{3+}) has been shown to be a substitute for Ca^{2+} in binding to several Ca^{2+}-binding proteins (Dockter, 1983; Leavis, Nagy, Lehrer, Bialkowska & Gergely, 1980). Phosphorescence and fluorescence intensity of Tb^{3+} increases upon binding to Ca^{2+}-binding sites and it can therefore act as a reporter group for the study of the characteristics of Ca^{2+}-binding sites. Purified PA IVb was examined at room temperature. PA was dissolved in a stock solution containing 20 mM-MOPS (3-(*N*-morpholino)propanesulphonic acid), 150 mM-KCl, 1 mM-β-mercaptoethanol and pH 7·0. Stock [PA] was 0·073 mM.

The first type of steady-state experiment was Tb^{3+} titration of PA. Phosphorescence signals were monitored by a Perkin–Elmer fluorescence spectrophotometer set in phosphorescence mode with 230 nm excitation and 545 nm emission. Tb^{3+} titrations of 5 μM-PA IVb were performed in 1 ml buffer solution (150 mM-KCl, 20 mM-MOPS and pH 7·0). Control experiments were performed by the addition of equal amounts of Tb^{3+} to the same buffer in the absence of PA.

The second type of steady-state experiment was Tb^{3+} displacement from PA by Mg^{2+} and Ca^{2+}. PA (0·074 or 0·09 μM) in 1 ml buffer solution (150 mM-KCl, 20 mM-MOPS and pH 7·0) was saturated by addition of 0·2 μM-$TbCl_3$. Tb^{3+} phosphorescence was recorded as Ca^{2+} was increased from 2·5 μM to 1 mM or as Mg^{2+} was increased from 200 to 700 mM. Control experiments with KCl substituted for Mg^{2+} indicated that none of the displacement of Tb^{3+} from PA by Mg^{2+} was due to ionic strength effects.

Kinetic experiments using dialysed PA

In order to reduce the level of Ca^{2+} contamination of PA, 18 ml of buffer solution containing 15–20 μM-PA, 5 mM-EDTA, 72 μM-β-mercaptoethanol was dialysed against 2 l of buffer solution at 5 °C. After 8 h the buffer solution outside the dialysis tubing was discarded and replaced with fresh buffer solution. The entire procedure used 6 l of buffer solution. The last dialysis buffer, calculated to contain 72 μM-β-mercaptoethanol and $3{\cdot}6 \times 10^{-9}$ M-EDTA, was used in subsequent kinetic experiments. PA buffer solution after dialysis contained 15–20 μM-PA, 72 μM-β-mercaptoethanol and $3{\cdot}6 \times 10^{-9}$ M-EDTA.

Ca^{2+} and Mg^{2+} dissociation rates from PA IVb were determined at 0 °C using an Applied Photophysics stopped-flow spectrometer (Model SF·17MV, Leatherhead, UK) which has a mixing time of 2 ms. The stopped-flow apparatus was thermostatically controlled with an accuracy of $\pm 0{\cdot}1$ °C and was used in the fluorescence mode with excitation at 250 nm and emission detected through a 550 nm interference filter. Stopped-flow measurements were made by rapidly mixing equal volumes of two solutions (A and B) and monitoring the time course of the fluorescence change. For each set of experiments, five traces were averaged and fitted to a single exponential equation for determination of the rate constant. Ca^{2+} dissociation rates from PA were determined by mixing solution A (containing 20 μM-PA IVb plus 500 μM-Ca^{2+}) with solution B (containing 500 μM-$TbCl_3$). Mg^{2+} dissociation rates were determined by mixing solution A (containing 20 μM-PA IVb plus either 10 or 100 mM-Mg^{2+}) with solution B (containing 500 μM-$TbCl_3$). The buffer in each case was that used for the last dialysis of PA and contained 20 mM-MOPS, 150 mM-KCl, 72 μM-β-mercaptoethanol, $3{\cdot}6 \times 10^{-9}$ M-EDTA, at pH 7·0. In control experiments dialysis buffer in the absence of PA was mixed with 500 μM-$TbCl_3$ and no time-dependent fluorescent change was observed. These control traces were subtracted from the traces obtained in the presence of PA. Tb^{3+} reaction with apo-PA (metal free) (as monitored by its fluorescence change) occurred within the 2 ms mixing time of the stopped-flow instrument consistent with the results of Leavis *et al.* (1980) for Tb^{3+} on-rates to apo-troponin C.

Statistical analysis

All results are presented as means $\pm$ S.D. Linear and non-linear regressions were calculated by the least-squares method. Student's t test and a paired t test were used for determination of significance. A significance level of $P \leqslant 0{\cdot}05$ was accepted. Results were excluded if the parameter under consideration was greater than 3 S.D.s from the mean calculated with the parameter omitted.

RESULTS

PA identification and concentration determination in fibres

One-dimensional SDS PAGE was used to identify and quantify PA in single fibres. Figure 1 shows an example of a gel in which several amounts of PA IVa (lanes 1–5) and IVb (lanes 6–10) standards and three typical single fibres (lanes 11–13) were run. Conditions of the gel run separated proteins and peptide subunits in the molecular weight range of 200 kDa (myosin heavy chain) to < 11 kDa (PAs). PA was identified by comparison to standards. Every fibre analysed exhibited both isoforms of PA. Even though the molecular weights of the PA isoforms are nearly identical (PA IVa, 11·6 kDa and PA IVb, 11·7 kDa (Ogawa & Tanokura, 1986*a*)), they are well

separated under these gel conditions. Figure 2 shows an example of a densitometric scan of the three fastest migrating bands for one of the fibres (lane 13) in Fig. 1. The PA bands are well separated from each other and from myosin light chain 3. Two-dimensional PAGE (Fig. 3) of bundles of frog fibres verified that each band attributed to PA contained only one protein.

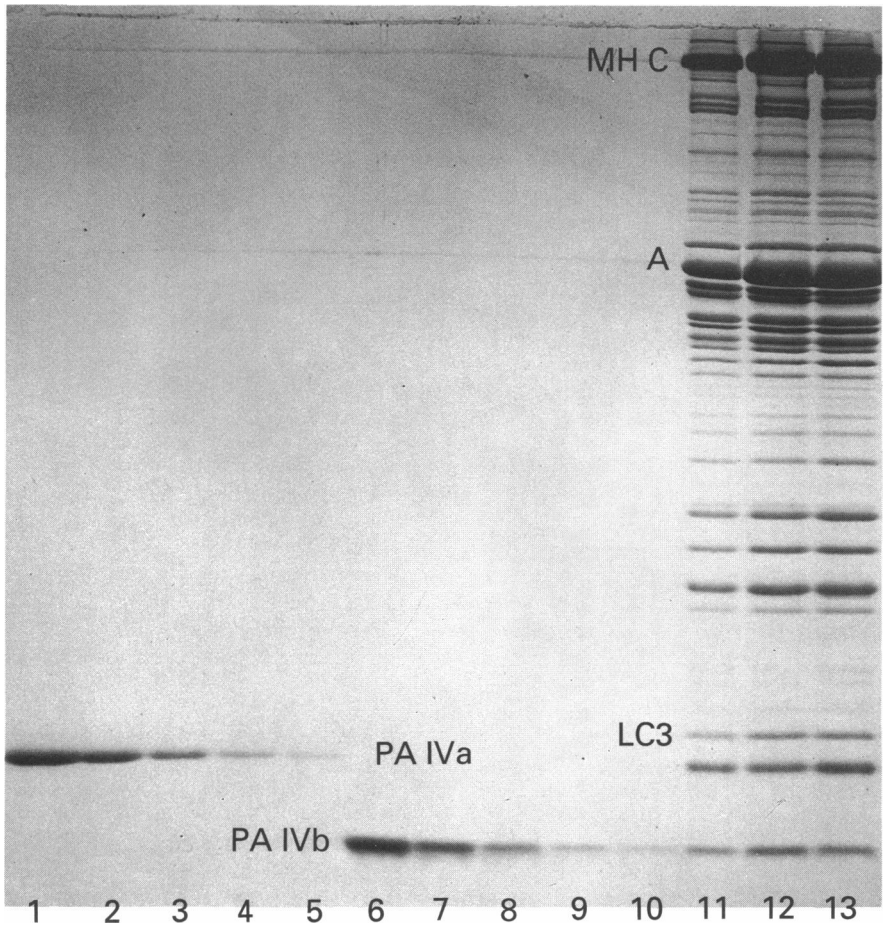

Fig. 1. One-dimensional SDS PAGE of single tibialis anterior fibres and purified PA standards from *Rana temporaria*. PA IVa standards (lanes 1–5) are 0·88, 0·44, 0·22, 0·11, and 0·055 μg respectively and PA IVb standards (lanes 6–10) are 0·74, 0·37, 0·185, 0·093, and 0·046 μg respectively. Bands for three typical fibres (lanes 11–13) range from 200 kDa (myosin heavy chain, MHC) to 11·5 kDa (PA). A locates actin and LC3 locates myosin light chain 3.

The [PA] was determined in twenty-five single fibres using calibration curves for known amounts of purified PA IVa and PA IVb which were linear in the range 45 ng to 0·9 μg. Average correlation coefficients for slopes of the calibration curves are $0{\cdot}991 \pm 0{\cdot}012$ ($n = 7$) for PA IVa and $0{\cdot}999 \pm 0{\cdot}001$ ($n = 7$) for PA IVb. Mean values of the slopes of calibration curves are not significantly different and the ratio of

slopes for PA IVa to IVb is 0·90. Mean quantity of total PA is $0·61 \pm 0·33$ μg and PA IVa represents 65 ± 11 % of total PA. Mean fibre volume is $0·099 \pm 0·050$ mm^3 and cross-sectional area at a resting sarcomere length of 2·2 μm is $14·6 \pm 6·8 \times 10^{-3}$ mm^2 (range $4·8–28·5 \times 10^{-3}$ mm^2). Thus mean total [PA] is $0·76 \pm 0·20$ mmol PA l^{-1} myoplasmic water.

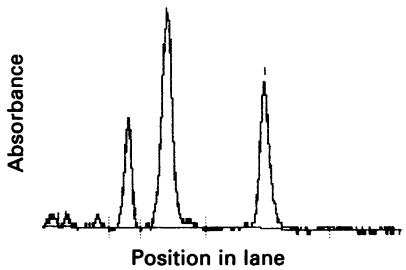

Fig. 2. Densitometric scan of the three fastest migrating bands in a typical gel of a single muscle fibre. This scan of absorbance *versus* position is of lane 13 of Fig. 1. Bands from left to right are: myosin light chain 3 (based on comparison to purified frog myosin), PA IVa (height of 0·14 absorbance units) and PA IVb. Calibration curves derived from standards run on the same gel indicated that this fibre contained 0·33 μg of PA IVa and 0·21 μg of PA IVb.

Time course of slowing of relaxation rate with increasing tetanus duration

Rate of relaxation ($1/t_{95–80\%}$) from an isometric tetanus slowed progressively with increasing tetanus duration over the range 0·3–5 s. This result is seen in Fig. 4*A* where tetani of 0·3, 2 and 5 s have been superimposed and force has been normalized to the force at the time of the last stimulus in order to show more clearly the decreased rate of relaxation with increasing tetanus duration. After a 5 s tetanus, there was no further slowing of relaxation rate in tetani as long as 15 s in duration (Fig. 4*B*). The relaxation rate decreases in an exponential manner as a function of tetanus duration as shown in Fig. 4*C* for a typical fibre. Results from each of seventeen fibres were fitted with an exponential equation of the form: $R = R_0 e^{-t\tau_r} + R_s$ where R is relaxation rate, R_0 is initial 'extra' rate of relaxation at $t = 0$, τ_r is rate constant for the effect, and R_s is steady value of relaxation rate (Peckham & Woledge, 1986). Mean values are: $\tau_r = 1·18 \pm 0·35$ s^{-1}; $R_0 = 2·89 \pm 0·79$ s^{-1}; $R_s = 2·22 \pm 0·33$ s^{-1}. Thus relaxation rate decreases with increasing tetanus duration at a rate of 1·18 s^{-1}. Relaxation rate for a 0·3 s tetanus (shortest tetanus in which peak force is reached) is about two times faster than the value of relaxation rate for a 4 s tetanus. Slowing of relaxation rate with increasing tetanus duration probably is not associated with the fall in peak tetanus force since greater than 99 % of the slowing of relaxation rate occurs with only $4·7 \pm 1·3$ % ($n = 14$) decrease in peak tetanus force in a 4 s tetanus.

Other parameters of relaxation rate also were found to slow with increasing tetanus duration. Mean rate constants for exponential slowing of relaxation rate with increasing tetanus duration are: $1/t_{5\%} = 0·65 \pm 0·15$ s^{-1} ($n = 17$) and $1/t_{50\%} = 0·81 \pm 0·27$ s^{-1} ($n = 15$). The exponential phase of relaxation (ξ_{exp}) did not change systematically in tetani of $\leqslant 1$ s duration but slowed exponentially in tetani of 1–5 s

duration ($0{\cdot}93 \pm 0{\cdot}35$ s^{-1}, $n = 10$). Thus all measures of the time course of exponential slowing of relaxation rate are within a factor of two and indicate a pronounced reduction of muscle relaxation rate with increasing tetanus duration which occurs with a rate of about 1 s^{-1}.

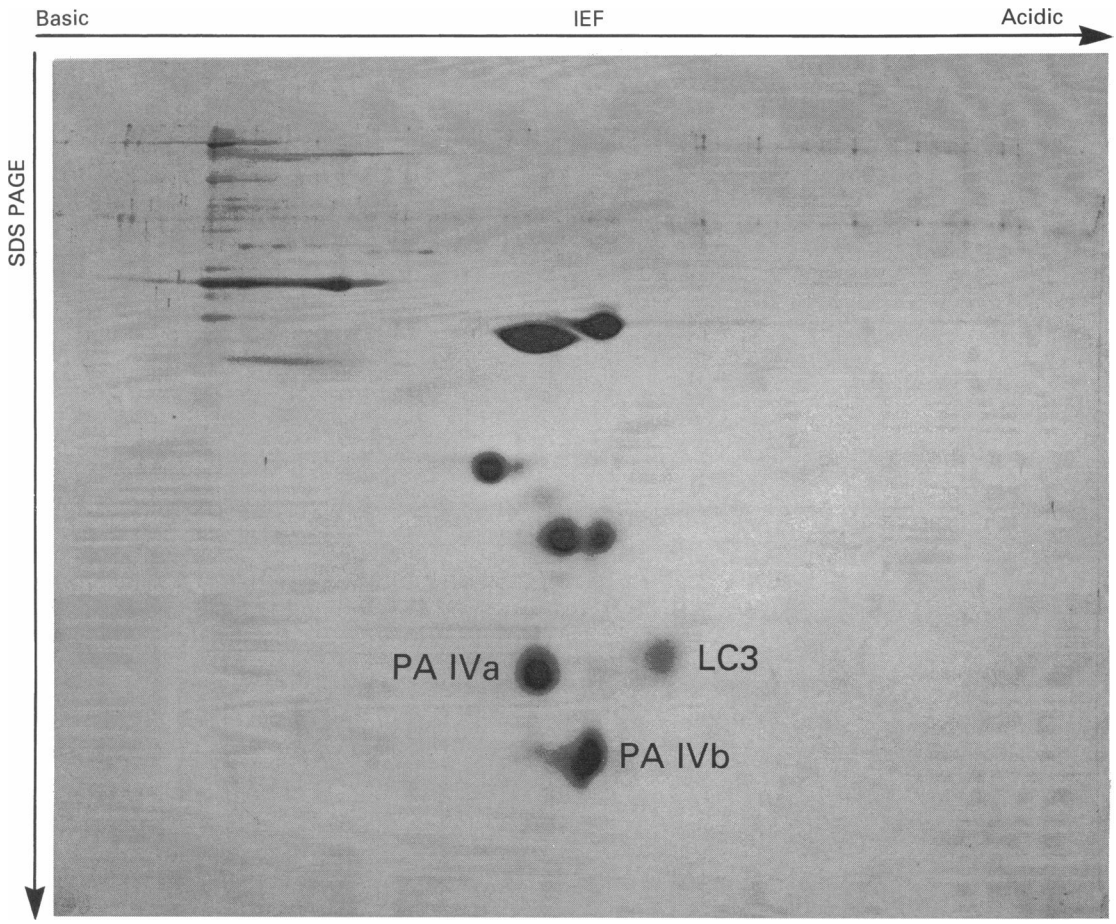

Fig. 3. Two-dimensional gel of a bundle of fibres from tibialis anterior of *Rana temporaria*. A bundle of fibres of 250 mg was loaded onto the gel. Separation by isoelectric focusing (IEF) occurred in the first dimension (horizontal) and separation according to molecular size (SDS PAGE) occurred in the second dimension (vertical). The gel is oriented with the basic side to the left and the acidic side to the right. Based on the migration pattern, the pI for PA IVa is greater than that for PA IVb, as expected (Gosselin-Rey & Gerday, 1977). Myosin light chain 3 is labelled as LC3. Two-dimensional gels of PA standards (not shown) displayed the same relative positions as the PA spots in this gel.

Recovery of relaxation rate after a prolonged tetanus with increasing rest interval

After a 4 s conditioning tetanus, relaxation rate ($1/t_{95\text{-}80\,\%}$) in a 0·5 s test tetanus increased with increasing rest intervals of 0·8, 5 and 30 s as shown in the examples in Fig. 5*A*. Results from a typical fibre shown in Fig. 5*B* indicate that recovery of

relaxation rate was exponential with increasing rest interval. Results were fitted with the equation: $R_n = R_i + R_e (1-e^{-t\kappa_r})$, where R_n is the ratio of relaxation rate in a test tetanus divided by relaxation rate in the conditioning tetanus, R_i is the ratio of relaxation rates at zero time interval between tetani which is taken as $t = 0$, R_e is the

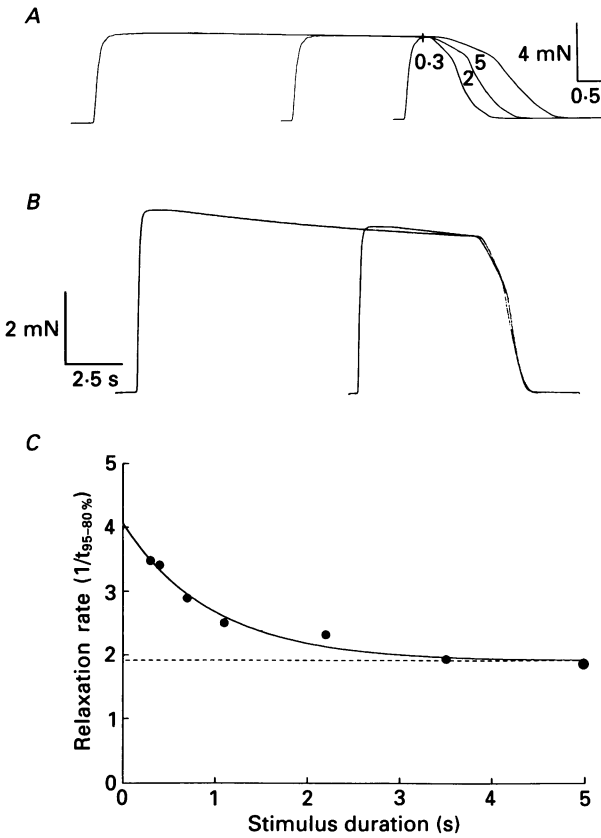

Fig. 4. Effects of isometric tetanus duration on time course of relaxation. *A*, relaxation rate slows progressively when tetanus duration varies from 0·3 to 2 to 5 s. *B*, there is little further change in relaxation rate when a 5 and 15 s tetanus are compared. In *A* and *B* records have been superimposed and force normalized to force at the last stimulus in order to show clearly changes in time course of relaxation. *C*, example of time course of slowing of relaxation rate with increasing tetanus duration. Relaxation rate slows exponentially with increasing tetanus duration and for this fibre: $R = 2{\cdot}15\, e^{-1{\cdot}04t} + 1{\cdot}91$.

magnitude of the effect and κ_r is the rate constant. Mean values ($n = 14$) are: $\kappa_r = 0{\cdot}12 \pm 0{\cdot}02\ s^{-1}$; $R_e = 0{\cdot}55 \pm 0{\cdot}17$ and $R_i = 0{\cdot}95 \pm 0{\cdot}03$. Mean value for relaxation rate of the conditioning tetani is $2{\cdot}58 \pm 0{\cdot}50\ s^{-1}$. Since the ratio of R_n after a 30 min rest period to $(R_e + R_i)$ ($0{\cdot}99 \pm 0{\cdot}09$, $n = 12$) is not significantly different from 1·0, the recovery process is adequately explained by a single exponential function. Thus relaxation rate recovers at a rate of $0{\cdot}12\ s^{-1}$.

The final relaxation rate of the 0·5 s test tetanus is $1{\cdot}6 \pm 0{\cdot}2$ times faster than the relaxation rate of the 4 s conditioning tetanus, i.e. $(R_e + R_i)/R_i$, with a mean absolute

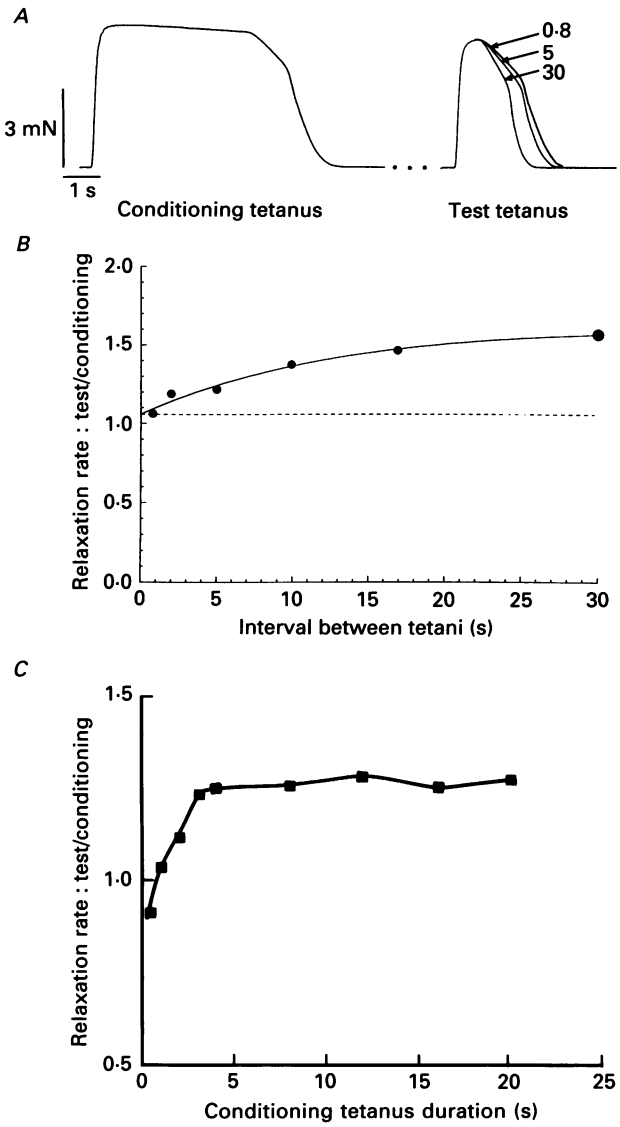

Fig. 5. Recovery of relaxation rate after a tetanus. *A*, superimposed examples of recovery of relaxation rate in a 0·5 s test tetanus after rest intervals of 0·8 to 30 s after conditioning tetani of 4 s duration. Although conditioning and test tetani were given in pairs, only one conditioning tetanus is shown. *B*, an example of the time course of recovery of relaxation rate with increasing rest interval after a prolonged tetanus. The value of relaxation rate of the test tetanus divided by relaxation rate of the conditioning tetanus increases exponentially after a 4 s conditioning tetanus as a function of duration of rest interval and for this fibre: $R_n = 0{\cdot}56\ (1-e^{-0{\cdot}08t}) + 1{\cdot}06$. *C*, an example of time course of recovery of relaxation rate in a 0·5 s test tetanus compared to that in a conditioning tetanus after a fixed rest interval of 5 s as influenced by duration of conditioning tetani from 0·5 to 20 s.

value of $1{\cdot}6 \times 2{\cdot}58\ s^{-1} = 4{\cdot}1\ s^{-1}$. This result can be compared to the relaxation rate of a 0·5 s tetanus which has not been preceded by a conditioning tetanus which is $1{\cdot}7 \pm 0{\cdot}2$ times faster than the final relaxation rate, i.e. $(R_0 e^{-0{\cdot}5\,\tau_r} + R_s)/R_s$, with a mean absolute value of $3{\cdot}8\ s^{-1}$. Since these relative relaxation rates are not significantly different, recovery of relaxation rate appears to be complete within the 30 s rest interval.

Effects of conditioning tetanus duration on the magnitude of recovery of relaxation rate in a test tetanus were examined. Figure 5*C* shows a plot of the magnitude of recovery of relaxation rate in a test tetanus compared to that in a conditioning tetanus after a 5 s rest interval *versus* conditioning tetani of various durations. Magnitude of recovery increased until the conditioning tetanus was approximately 4 s in duration and remained unaltered by conditioning tetanus duration from 4 to 20 s. This result confirms that a 4 s conditioning tetanus is necessary for the recovery experiments and suggests that longer conditioning tetani would not alter the results.

Other parameters of relaxation rate were determined as a function of rest interval between conditioning and test tetani. Mean rate constants for the exponential time course of recovery of relaxation rate are: $1/t_{5\%} = 0{\cdot}06 \pm 0{\cdot}01\ s^{-1}$ ($n = 14$), $1/t_{50\%} = 0{\cdot}07 \pm 0{\cdot}02\ s^{-1}$ ($n = 12$) and $\xi_{exp} = 0{\cdot}06 \pm 0{\cdot}03\ s^{-1}$ ($n = 9$). Thus all measures of time course of recovery of relaxation rate are within a factor of two and indicate that recovery of relaxation rate increases as rest interval increases with a rate of about $0{\cdot}1\ s^{-1}$.

PA concentration compared to magnitude of changes in relaxation rate

Figure 6 shows a plot of total [PA] in seven fibres compared to the magnitude of recovery of relaxation rate, R_e, in those same fibres. The [PA] was determined from three SDS gels. The coefficient of variation (S.D./mean) of the slopes of calibration curves from these gels was 7·6% for PA IVa and 9·5% for PA IVb. The slope of R_e *versus* total [PA] was significantly different from zero with a positive correlation coefficient of $r = 0{\cdot}80$ ($n = 7$). Thus fibres with higher [PA]s exhibit greater magnitudes of recovery of relaxation rate after a prolonged tetanus. A similar analysis was carried out comparing the magnitude of slowing of relaxation rate with increasing tetanus duration (R_0), to total [PA]. Contrary to the predictions of the hypothesis that PA promotes relaxation, linear regression analysis indicated that the slope relating these parameters was not significantly different from zero ($r = 0{\cdot}074$, $n = 6$). Slopes of other measures of changes of relaxation rate with tetanus duration or recovery interval versus [PA] were not significantly different from zero.

Determination of steady-state and kinetic properties of Ca^{2+} and Mg^{2+} interaction with PA

Ca^{2+} and Mg^{2+} binding to purified PA was examined using Tb^{3+} as a reporter group. Steady-state characteristics of Tb^{3+} binding to PA IVb were determined. Figure 7 shows an average ($n = 2$) titration curve of Tb^{3+} phosphorescence *versus* moles of Tb^{3+} added per mole of PA IVb at 23 °C. The curve was corrected for Tb^{3+} phosphorescence in the absence of PA IVb. Phosphorescence reached a peak when the Tb^{3+}-to-PA IVb ratio was near 2 mol mol^{-1}. This result is consistent with two

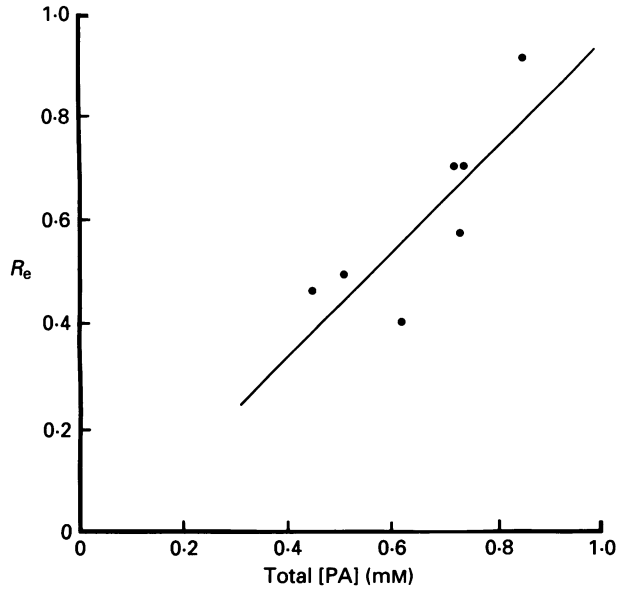

Fig. 6. Plot of the magnitude of recovery of relative relaxation rate after a prolonged tetanus (R_e) *versus* total PA concentration in single muscle fibres. For each fibre, R_e was determined, fibre volume was measured and then PA content was measured. The coefficient of variation (S.D./mean) for absorptance of a 0·5 μg PA standard among the three gels used was 5% for PA IVa and 12·6% for PA IVb. The line represents the best fit derived from linear regression analysis ($r = 0{\cdot}8$). For these fibres, mean values were: [PA], 0·7 mM; R_e, 0·6; τ_k, 0·12 s^{-1}; R_1, 0·94 and conditioning tetanus relaxation rate was 2·44 s^{-1}.

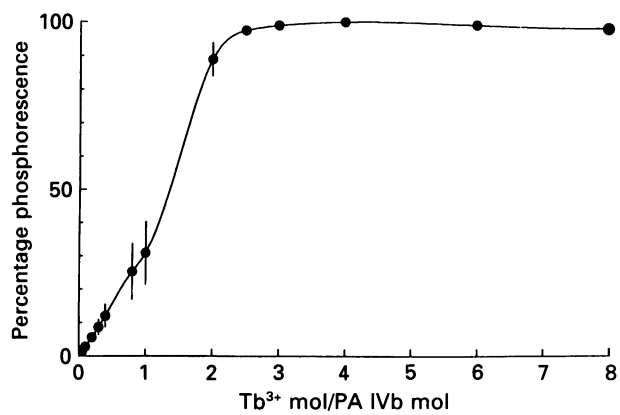

Fig. 7. Tb^{3+} titration of 5 μM-PA IVb at 23 °C. The curve was corrected for non-bound Tb^{3+} phosphorescence by subtracting Tb^{3+} luminescence in the absence of PA from Tb^{3+} luminescence plus PA at each Tb^{3+} concentration. 100% phosphorescence change corresponds to an approximately seventyfold increase in Tb^{3+} luminescence in the presence of PA over Tb^{3+} luminescence in the absence of PA. Experimental conditions are described in the methods section.

Tb^{3+} binding sites on PA IVb. The fact that Tb^{3+} binds to the Ca^{2+}/Mg^{2+} binding sites on PA IVb was confirmed by the observation that Ca^{2+} and Mg^{2+} displaced Tb^{3+} from PA IVb which resulted in a decrease in Tb-PA luminescence. Ca^{2+} was able to half-maximally displace 2 μM-Tb^{3+} from 0·074 μM-PA IVb at 55 μM whereas Mg^{2+}

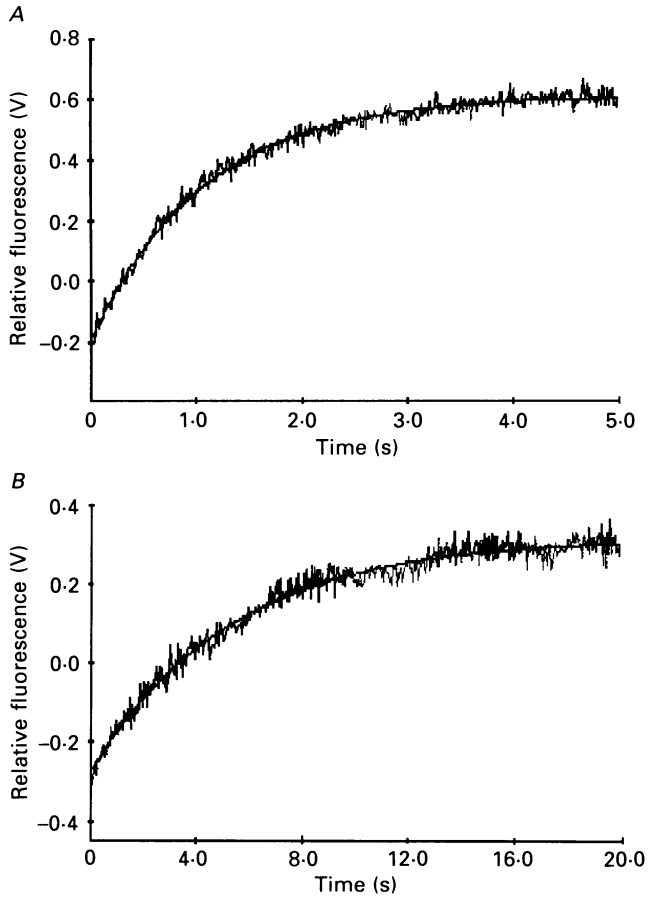

Fig. 8. The time course of Tb^{3+} fluorescence increase upon the addition of 500 μM-Tb^{3+} to purified PA IVb (20 μM) saturated with either 10 mM-Mg^{2+} (*A*) or 500 μM-Ca^{2+} (*B*) at 0 °C. In each case five traces were averaged and the resulting curves fitted with single exponential relationships. Rate constant for Tb^{3+} binding to PA-Mg is 0·93 s^{-1} and to PA-Ca is 0·19 s^{-1} (see Table 1).

was much less effective (half-maximal displacement was observed at 330 mM). These results are consistent with a Ca^{2+} affinity for PA which is about 6000 times greater than the Mg^{2+} affinity for PA.

The time course of Tb^{3+} binding to PA saturated with either Mg^{2+} or Ca^{2+} measured indirectly the Mg^{2+} and Ca^{2+} dissociation rates from purified PA. The time course of Tb^{3+} binding to PA-Mg IVb is shown in Fig. 8*A*. These data (average of five runs) are best fitted with a single exponential relation with a rate constant of 0·93 s^{-1} (see Table 1). The Mg^{2+} dissociation rate from PA IVb was not dependent on Mg^{2+} concentration

($[Mg^{2+}]$) in the range 10–100 mM-Mg^{2+} and was not significantly different from that observed for PA IVa (data not shown). The time course of Tb^{3+} binding to PA–Ca IVb is shown in Fig. 8*B*. These data (average of five runs) are best fitted with a single exponential relation with a rate constant of 0·19 s^{-1} (see Table 1). The Ca^{2+} dissociation rate from PA IVb was not dependent on Ca^{2+} concentration ($[Ca^{2+}]$) in

TABLE 1. Summary of mean rate constants (±S.D.) for slowing and recovery of relaxation rate in single fibres from anterior tibialis muscles of *Rana temporaria* and Mg^{2+} and Ca^{2+} dissociation rates from purified PA IVb from *Rana temporaria* at 0 °C

Rate of slowing of relaxation rate (s^{-1})	Rate of recovery of relaxation rate (s^{-1})	Mg^{2+} dissociation rate from PA (s^{-1})	Ca^{2+} dissociation rate from PA (s^{-1})
1·18±0·35 ($n = 17$)	0·12±0·02 ($n = 14$)	0·93±0·02 ($n = 5$)	0·19±0·01 ($n = 5$)

the range of 0·5–2 mM-Ca^{2+} and was not significantly different from that observed for PA IVa (data not shown).

Table 1 summarizes results for time course measurements of slowing of rate of relaxation with increasing tetanus duration and recovery of relaxation rate with increasing rest interval after a prolonged tetanus in single fibres and compares these results to the Mg^{2+} and Ca^{2+} dissociation rates from purified PA. The Mg^{2+} dissociation rate from PA (0·93 s^{-1}) is very similar to (but significantly less than) the rate constant for the time course of slowing of relaxation rate with increasing tetanus duration (1·18 s^{-1}). The Ca^{2+} dissociation rate from PA (0·19 s^{-1}) is similar to (but significantly greater than) the rate constant for recovery of relaxation rate after a prolonged tetanus (0·12 s^{-1}).

DISCUSSION

Parvalbumin concentration [PA] in single skeletal muscle fibres

Total [PA] in single fibres, isolated from tibialis anterior muscles of *Rana temporaria*, averaged 0·76 mM. This mean is greater than the value of approximately 0·52 mM (reported as 0·3 μmol g^{-1} wet wt) observed for whole leg muscles of *Rana temporaria* (Gosselin-Rey & Gerday, 1977). The lower value of [PA] in whole leg muscle may be due to (a) incomplete extraction of PA and/or (b) differences in [PA] in different fibre types of whole leg muscles. Mean cross-sectional area of fibres in this study ($14{\cdot}6 \times 10^{-3}$ mm^2) is similar to the mean ($13{\cdot}0 \times 10^{-3}$ mm^2) reported for large fast-twitch fibres of *Rana temporaria* (Elzinga, Howarth, Rall, Wilson & Woledge, 1989).

It is of interest to compare the *total* myoplasmic $[Ca^{2+}]$, measured during rest and during a tetanus, to the calculated total Ca^{2+}-binding capacity of a fibre. Total myoplasmic $[Ca^{2+}]$ in the I-band of *Rana pipiens* skeletal muscle is 0·6–0·8 mmol l^{-1} fibre water during rest (Somlyo, McClellan, Gonzalez-Serratos & Somlyo, 1985) and increases to about 1 mM during a tetanus (Somlyo, Gonzalez-Serratos, Shuman, McClellan & Somlyo, 1981). The calculated total Ca^{2+}-binding capacity, [TN] × four binding sites per molecule + [PA] × two binding sites per molecule, is 1·9 mM. (TN contains two Ca^{2+}-specific regulatory sites and two Ca^{2+}/Mg^{2+}-binding sites and [TN]

in skeletal muscle is 0·09 mM (Yates & Greaser, 1983).) Thus the total Ca^{2+}-binding capacity of a fibre is not exceeded during a prolonged tetanus. The total myoplasmic $[Ca^{2+}]$ at rest suggests that under these conditions about 60% of the Ca^{2+}/Mg^{2+}-binding sites contain Mg^{2+} and 40% contain Ca^{2+}.

Relationship of time course of slowing of relaxation rate with increasing tetanus duration to Mg^{2+} dissociation from PA

If PA promotes relaxation, the relaxation rate should decrease exponentially with increased tetanus duration because the elevated Ca^{2+} during a tetanus would bind to PA and thus reduce the capacity of PA to bind Ca^{2+} during relaxation. The observation that the rate of decay of the Ca^{2+} transient is slowed with increasing tetanus duration in *Rana temporaria* skeletal muscle at 10 °C is consistent with this prediction (Blinks *et al.* 1978; Cannell, 1986). Furthermore there is a positive correlation between rate of decay of Ca^{2+} transient and rate of relaxation (measured as $1/t_{95\text{-}80\%}$; Cannell, 1986). In the present study, relaxation rate (measured as $1/t_{95\text{-}80\%}$) decreased exponentially with increasing tetanus duration with a mean rate constant of 1·18 s^{-1} at 0 °C (Table 1). This rate constant is greater than values reported for the exponential time course of slowing of relaxation rate (measured as $1/t_{5\%}$) at 0 °C of 0·4 and 0·3 s^{-1} in whole sartorius and extensor longus digiti IV muscles of *Rana temporaria* respectively (Peckham & Woledge, 1986). Much of the difference appears to be due to the parameter employed to assess relaxation rate since $1/t_{5\%}$ averaged 0·65 s^{-1} in the present study. As Peckham & Woledge (1986) have emphasized, $1/t_{5\%}$ could tend to underestimate the rate constant for slowing of relaxation rate because it includes a portion of relaxation that may be limited by a fixed delay due to cross-bridge detachment. The relative contribution of the fixed delay to measures of relaxation rate would be greater in shorter tetani where relaxation rate is faster. The parameter $1/t_{95\text{-}80\%}$ would tend to minimize this complication and may reflect more accurately the decay of the Ca^{2+} transient.

If PA promotes relaxation, the time course of slowing of relaxation rate with increasing tetanus duration should be related closely to the rate of dissociation of Mg^{2+} from PA. This result is expected because [PA-Mg], which is directly related to the capacity of PA to buffer Ca^{2+} during relaxation and thus promote relaxation, decreases as Ca^{2+} exchanges for Mg^{2+} during a tetanus in a reaction which is limited by the rate of Mg^{2+} dissociation from PA. This overall reaction is:

$$\text{PA-Mg} + \text{Ca}^{2+} \rightleftharpoons \text{PA-Ca} + \text{Mg}^{2+} \tag{1}$$

and this reaction can be considered as the sum of two reactions:

$$\text{PA-Mg} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{PA} + \text{Mg}^{2+} \tag{2}$$

and

$$\text{PA} + \text{Ca}^{2+} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{PA-Ca}. \tag{3}$$

If the affinity (expressed as $K = k_{\text{association}}/k_{\text{dissociation}}$) of PA for Ca^{2+} is 10^4 times greater than that for Mg^{2+} ($K_{Ca} \sim 10^8$ M^{-1} and $K_{Mg} \sim 10^4$ M^{-1}; Potter *et al.* (1977)) and the ratio of Ca^{2+}-to-Mg^{2+} dissociation rates (k_{-2}/k_{+1}) is 0·2 ($k_{-2} \sim 0{\cdot}2$ s^{-1} and k_{+1}

$\sim 1\ s^{-1}$, Table 1), then the ratio of Ca^{2+}-to-Mg^{2+} association rates (k_{+2}/k_{-1}) to PA must be approximately 2000. Since the free myoplasmic [Mg^{2+}] is 1 mM (Alvarez-Leefmans, Gamino, Giraldez & Gonzalez-Serratos, 1986) and free [Ca^{2+}] about 10 μM (Blinks *et al.* 1978) during a tetanus, the probability that Mg^{2+} will rebind to PA after dissociation is only 5%. Furthermore the dissociation rates of Ca^{2+} (k_{-2}) and Mg^{2+} (k_{+1}) from PA are slow compared to the diffusion-limited association rate of Ca^{2+} (k_{+2}) to PA (Porter *et al.* 1978). Thus the overall reaction for Ca^{2+} association to PA-Mg should be determined by the rate of Mg^{2+} dissociation from PA (k_{+1}). Finally, the Mg^{2+} dissociation rate from PA would be expected to relate closely to the rate of slowing of relaxation rate with increasing tetanus duration in fibres if PA promotes muscle relaxation.

The observed value of the Mg^{2+} dissociation rate constant from purified PA measured under conditions similar to those in fibres (150 ionic strength, pH 7·0, 0 °C) averaged $0{\cdot}93\ s^{-1}$ (Table 1). This value is very close to, but significantly different from, the rate constant observed for the time course of slowing of relaxation in fibres ($1{\cdot}18\ s^{-1}$). None the less, given the different conditions, agreement within 30% between a physiological parameter and studies with purified proteins seems consistent with the interpretation that PA plays a role in promoting muscle relaxation. Also the present results predict that there should be a transient increase in intracellular free [Mg^{2+}] in a fibre during contraction with a rate constant of about $1\ s^{-1}$ at 0 °C. A Mg^{2+} transient with a rate constant of 3–4 s^{-1} has been observed during contraction of *Rana temporaria* skeletal muscle fibres at 18 °C (Irving, Maylie, Sizto & Chandler, 1989).

Mechanical experiments allow an estimate of magnitude of the relaxing effect of PA. The amplitude of the transient component of relaxation rate is such that relaxation rate in a 0·3 s a tetanus of tibialis anterior of *Rana temporaria* is about twice as great as the value in a tetanus of 4–15 s duration. Thus PA may speed up relaxation rate in this muscle at 0 °C by a factor of about two. According to these arguments, if tetanus duration is 4 s or longer at 0 °C, PA would play no role in relaxation presumably because it is saturated with Ca^{2+}.

Relationship of time course of recovery of relaxation rate after a prolonged tetanus to Ca^{2+} dissociation from PA

If PA promotes relaxation, the time course of recovery of relaxation rate after a prolonged tetanus should be closely related to the re-formation of PA-Mg in reaction (1) after a tetanus. The rate of re-formation of PA-Mg after a prolonged tetanus is expected to be limited by, and thus similar to, the rate of Ca^{2+} dissociation from PA (Robertson *et al.* 1981; Irving *et al.* 1989). Relaxation rate (measured as $1/t_{95-80\%}$) recovers exponentially after a prolonged tetanus with a mean rate constant of $0{\cdot}12\ s^{-1}$ at 0 °C (Table 1). A single exponential completely explains the results. This observation is at variance with results of Peckham & Woledge (1986) who observed that a sum of two exponentials was required to explain 80% of the recovery of relaxation rate at 0 °C in whole sartorius muscles with components of similar amplitudes and with rate constants of 0·05 and $0{\cdot}005\ s^{-1}$. The faster rate constant is similar to results reported here ($1/t_{5\%} = 0{\cdot}06\ s^{-1}$). Differences in results are unexplained but may relate to the different protocol employed to measure and to

induce changes in relaxation rate, i.e. Peckham & Woledge (1986) used 'interrupted' tetani.

Ca^{2+} dissociation rate from purified PA was exponential with a mean rate constant of 0·19 s^{-1} at 0 °C (Table 1). This value is very similar but significantly different to the observed rate constant for recovery of relaxation rate of 0·12 s^{-1}. None the less, given the different conditions, agreement within a factor of about 1·5 seems consistent with the interpretation that the time course of recovery of relaxation rate with rest after a prolonged tetanus (0·12 s^{-1}) is controlled by Ca^{2+} dissociation from PA (0·19 s^{-1}).

This interpretation predicts that myoplasmic Ca^{2+} should not return completely to SR until after 20 s (four × time constant for Ca^{2+} dissociation from PA) of rest at 0 °C. Consistent with this prediction, electron probe studies with *Rana pipiens* (20–23 °C) and *Rana temporaria* (0 °C) skeletal muscle have shown that Ca^{2+} does not return completely to SR until after relaxation (Somlyo *et al.* 1985; Tormey & Homsher, 1986). At 0 °C SR Ca^{2+} content does not recover to pre-tetanus values until 30 s after the last stimulus of a prolonged tetanus (5 s duration) (Tormey & Homsher, 1986). Thus electron probe results are compatible with the interpretation that Ca^{2+} dissociation from PA limits rate of Ca^{2+} sequestration by the SR.

Relationship of [PA] to magnitude of changes in relaxation rate

A prediction based on the model of PA as a relaxing factor is that the magnitude of change in relaxation rate should depend on [PA] in fibres. For recovery of relaxation rate after a prolonged tetanus (4 s), magnitude of recovery (R_e) correlated significantly with [PA] in the same fibres. Thus fibres with a higher [PA] exhibited greater magnitudes of recovery of relaxation rate. However, magnitude of slowing of relaxation rate with increasing tetanus duration (R_0) did not correlate significantly with [PA]. This result is not consistent with the model of PA as a relaxing factor and suggests that other factors along with PA may be involved in controlling relaxation rate. A general problem with this analysis is that it is PA-Mg that would be predicted to correlate with magnitude of change in relaxation rate and not necessarily total [PA].

Ca^{2+}/Mg^{2+} exchange on PA and absolute rate of relaxation

It is important to consider whether Ca^{2+}/Mg^{2+} exchange on PA is rapid enough to contribute to rate of relaxation in a tetanus in frog skeletal muscle at 0 °C. As an example, consider relaxation from a 0·5 s tetanus. In order for muscle to relax, Ca^{2+} bound to Ca^{2+}-specific sites of TN must be removed. Concentration of Ca^{2+}-specific sites of TN is 0·18 mM (Yates & Greaser, 1983). In frog muscle [PA] is 0·76 mM. If about half of the two Ca^{2+}/Mg^{2+}-binding sites on PA are occupied by Mg^{2+} at rest (see above), then [PA-Mg] is 0·76 mM. According to results for dissociation rate of Mg^{2+} from PA (1 s^{-1}), during 0·5 s of stimulation about 40% of PA-Mg exchanges for Ca-PA. Therefore about 60% of Mg-PA or 0·46 mM is present at the beginning of relaxation. Based on this information and the assumption that Ca^{2+} dissociation from Ca^{2+}-specific sites of TN (about 30 s^{-1} at 22 °C) is not rate limiting (Johnson, Robinson, Robertson, Schwartz & Potter, 1981), time course of Ca^{2+} removal from TN by PA-Mg during relaxation can be estimated as: $Y = [0{\cdot}18 - 0{\cdot}46\ (1 - e^{-1t})]/0{\cdot}18$

where Y is the fraction of Ca^{2+}-specific sites of TN occupied by Ca^{2+} at time t. Time courses of a 0·5 s tetanus at 0 °C and predicted Ca^{2+} removal from TN by PA-Mg during relaxation are shown in Fig. 9. The actual rate of decline of myoplasmic free $[Ca^{2+}]$ would be more rapid still since the SR is proposed to function in parallel with

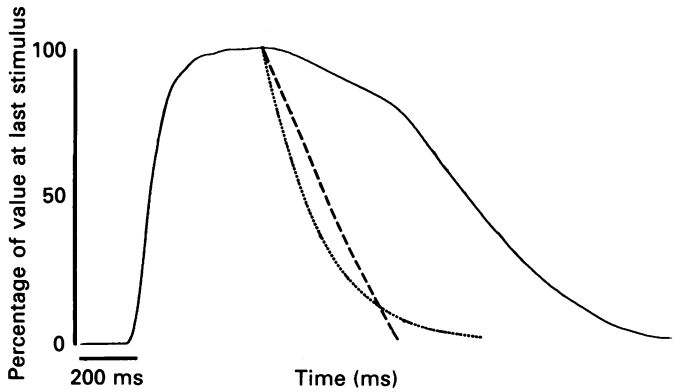

Fig. 9. Comparison of time courses of mechanical relaxation, calculated Ca^{2+} removal from TN by PA-Mg duration relaxation and estimated Ca^{2+} transient during relaxation in frog skeletal muscle at 0 °C. Continuous line, time course of a 0·5 s isometric tetanus (amplitude of 5·7 mN) in a tibialis anterior fibre from *Rana temporaria* (T.-t. Hou and J. A. Rall, unpublished observation). Dashed line, decline of the concentration of TN-Ca complex during relaxation expressed as a fraction of its maximal value (0·18 mM) just before force starts to decline (see text). Dotted line, relative decrease of the myoplasmic $[Ca^{2+}]$ from its maximal value (10 μM).

PA. An estimate of rate of decline of free $[Ca^{2+}]$ at 0 °C can be derived from observations that the rate of fall of free $[Ca^{2+}]$ during relaxation from a 0·5 s tetanus at 5 °C is about 9 s^{-1} with a temperature coefficient (Q_{10}) of 2·8 (Cannell, 1982). Thus free $[Ca^{2+}]$ should decline during relaxation in a 0·5 s tetanus at 0 °C with a rate constant of about 5 s^{-1}. This fall in free $[Ca^{2+}]$ can be compared to Ca^{2+} removal from TN by PA-Mg in Fig. 9. Based on this analysis, rate of Ca^{2+} binding by PA appears to be fast enough to contribute to the rate of relaxation from a 0·5 s tetanus at 0 °C.

In summary quantitative evidence supports the hypothesis that time courses of slowing and recovery of relaxation rate in isometric tetani of *Rana temporaria* skeletal muscle at 0 °C may be, to a large extent, controlled by Mg^{2+} and Ca^{2+} dissociation from PA respectively. Thus evidence supports a role for PA in facilitating relaxation in frog skeletal muscle at low temperature. Effectiveness of PA as a relaxing factor depends on tetanus duration and the interval between tetani. Four seconds of tetanic stimulation at 0 °C exhausts the ability of PA to promote relaxation and about 20 s of rest is required for maximum recovery of the relaxing effect. Uncertainties remain, particularly important are those associated with the fraction of PA that contains Mg^{2+} at rest.

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