Calcium Transients and the Effect of a Photolytically Released Calcium Chelator during Electrically Induced Contractions in Rabbit Rectococcygeus Smooth Muscle

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ABSTRACT Intracellular Ca^{2+} was determined with the fura-2 technique during electrically induced contractions in the rabbit rectococcygeus smooth muscle at 22°C. The muscles were electrically activated to give short, reproducible contractions. Intracellular [Ca^{2+}] increased during activation; the increase in [Ca^{2+}] preceded force development by ~2 s. After cessation of stimulation Ca^{2+} fell, preceding the fall in force by ~4 s. The fluorescence properties of fura-2 were determined with time-resolved spectroscopy using synchrotron light at the MAX-storage ring, Lund, Sweden. The fluorescence decay of free fura-2 was best described by two exponential decays (time constants ~0.5 and 1.5 ns) at low Ca^{2+} (pCa 9). At high Ca^{2+} (pCa 4.5), fluorescence decay became slower and could be fitted by one exponential decay (1.9 ns). Time-resolved anisotropy of free fura-2 was characteristic of free rotational motion (correlation time 0.3 ns). Motion of fura-2 could be markedly inhibited by high concentrations of creatine kinase. Time-resolved spectroscopy measurements of muscle fibers loaded with fura-2 showed that the fluorescence lifetime of the probe was longer, suggesting an influence of the chemical environment. Anisotropy measurements revealed, however, that the probe was mobile in the cells. The Ca^{2+}-dependence of contraction and relaxation was studied using a photolabile calcium chelator, diazo-2, which could be loaded into the muscle cells in a similar manner as fura-2. Photolysis of diazo-2 leads to an increase in its Ca^{2+}-affinity and a fall in free Ca^{2+}. When muscles that had been loaded with diazo-2 were illuminated with UV light flashes during the rising phase of contraction, the rate of contraction became slower, suggesting a close relation between intracellular Ca^{2+} and the cross-bridge interaction. In contrast, photolysis during relaxation did not influence the rate of force decay, suggesting that relaxation of these contractions is not determined by the rate of Ca^{2+} removal or due to an increased Ca^{2+} sensitivity, but instead is limited by other processes such as deactivation by dephosphorylation or detachment of tension-bearing cross-bridges, possibly regulated by thin filament systems.

INTRODUCTION

Activation of smooth muscle is associated with an increase in intracellular calcium (cf. Fay et al., 1979). In the cell, a Ca^{2+} calmodulin-dependent phosphorylation of regulatory light chains on the myosin molecule is considered to be the major mechanism for coupling the rise in free [Ca^{2+}] to force generation by the cross-bridge interaction between myosin and actin filaments (cf. review by Kamm and Stull, 1989). It has been shown that the relations between both [Ca^{2+}] and force (Yagi et al., 1991), and phosphorylation and force (Dillon et al., 1981), are not unique, but vary during the course of contraction in smooth muscle. This phenomenon has been interpreted to reflect the presence of force bearing dephosphorylated "latch" bridges formed by dephosphorylation of attached cross-bridges (Hai and Murphy, 1988). In addition to phosphorylation-dependent regulation of cross-bridges, other mechanisms have been proposed to influence the Ca^{2+}-phosphorylation-force coupling, e.g., attachment of dephosphorylated cross-bridges by cooperative mechanisms (Vyas et al., 1992; Somlyo et al., 1988) or regulation by thin filament-associated proteins (cf. Marston and Smith, 1985). The calcium sensitivity of phosphorylation is dependent, for example, on the mode of stimulation, showing that the relation between intracellular [Ca^{2+}] and myosin phosphorylation is also variable (Morgan and Morgan, 1984; Rembold, 1990; Himpens and Somlyo, 1988). This modulation of the Ca^{2+} sensitivity of phosphorylation could tentatively be a result of alterations in the myosin light chain kinase (MLCK) or myosin light chain phosphatase (MLCP) activities. On the basis of results from α-toxin permeabilized smooth muscle it has been proposed that G-protein-mediated processes can be involved in the agonist-mediated modulation of Ca^{2+} sensitivity (Fujiwara et al., 1989; Kitazawa et al., 1989, 1991; Nishimura et al., 1988). This effect in agonist-induced contractions is considered to be caused by modulation of phosphatase activity, possibly via action on small G-proteins (Somlyo and Somlyo, 1994; Otto et al., 1996). In a study on spontaneous contractions in uterine smooth muscle, Word et al. (1994) reported that the Ca^{2+} sensitivity of the MLCK was initially high and decreased during the course of contraction, which suggests that the rate of phosphorylation also can be mediated by changes in the Ca^{2+} sensitivity of the kinase activity.

Received for publication 15 January 1998 and in final form 25 May 1998.
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This paper is dedicated to our friend and colleague Dr. Frederic S. Fay.

0006-3495/98/10/1895/09 $2.00

In the intact smooth muscle the relation between [Ca^{2+}] and phosphorylation/force is nonlinear, and is also complicated by the comparatively slow kinetics of the activation processes and of the cross-bridge interaction. Therefore, alterations in the relation between [Ca^{2+}] and active force during different phases of contraction cannot be directly interpreted as changes in Ca^{2+} sensitivity of the contractile system. To directly test whether the Ca^{2+} sensitivity of force changes during contraction we have used caged-compound technology to momentarily alter the intracellular Ca^{2+} concentration during different phases of contraction in a smooth muscle.

We have utilized the "caged" calcium chelator, diazo-2 (Adams et al., 1989). The Ca^{2+} affinity of the native form of this compound is low ($K_d \sim 2.2$ μM), but after an intense UV light flash it is converted to a high-affinity Ca^{2+} binding form ($K_d \sim 0.07$ μM) and can therefore be used to rapidly lower the free Ca^{2+} concentration. Diazo-2 has been used to study the relaxation process in skinned striated and smooth muscle fibers (Palmer et al., 1990; Khromov et al., 1995). The compound is available in an acetoxymethyl ester form (diazo-2/AM) and can be loaded into intact fibers. In living striated muscle, flash photolysis of diazo-2 can increase the rate of relaxation after a short tetanus (Lännergren and Arner, 1992). In the present study we have loaded diazo-2 into rabbit rectococcygeus smooth muscle fibers and photolyzed the compound during different phases of electrically induced contractions.

To characterize the relation between Ca^{2+} and force in the rectococcygeus muscle preparation we used the fura-2 technique. A potential problem with measurements using fluorescent probes is binding to intracellular proteins, which might influence the spectroscopic properties and the cellular distribution of the probe. Keating and Wensel (1991) have reported, using time-resolved fluorescence microscopy, that in rat basophilic leukemic cells fura-2 can be partially immobilized by binding to intracellular macromolecules. In an early study using steady-state polarization measurements on fura-2 in isolated single smooth muscle cells, no indication for a restricted motion was found (Williams et al., 1985). Detailed information on the rotational diffusion of Ca^{2+} indicators in smooth muscle cells is, however, very sparse. We have therefore used a synchrotron light source and determined the time-resolved fluorescence and anisotropy of fura-2 salt at high and low [Ca^{2+}] and in the presence of protein at high concentrations. Measurements were also performed on rectococcygeus muscle fibers that had been loaded with fura-2 to obtain information regarding the rotational diffusion of the probe in the smooth muscle cells.

METHODS

Preparation and solutions

Small strips (length ~5 mm, diameter 0.1–0.3 mm) were excised from rabbit rectococcygeus muscles and studied at 22°C in a bicarbonate-buffered physiological salt solution (Krebs solution) of the following composition (in mM): NaCl 112, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.5, EDTA 0.026, gassed with 96% O_2 and 4% CO_2, giving a pH of ~7.4. Ca^{2+}-free solution was made by omitting $CaCl_2$. Loading of fura-2 was performed at 22°C by keeping muscle strips in a Ca^{2+}-free Krebs solution containing 10 μM fura-2/AM (Molecular Probes, Eugene, OR), 1% DMSO (dimethyl sulfoxide), and 0.02% Pluronic F-127 (BASF from Molecular Probes) for 2–3 h. After the loading period the muscle preparations were kept in Krebs solution for ~1 h prior to the experiments. For loading of diazo-2, the same procedure was used except that fura-2/AM was replaced by 10 μM diazo-2/AM (Molecular Probes). Experiments on free fura-2 (added as pentapotassium salt) were performed in a solution containing (in mM): TES (*N*-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid) 30.0, Mg^{2+} 2.0, MgATP 1.0, ionic strength (adjusted with KCl) 150, phosphocreatine 5.0, at pH 6.9 (adjusted with KOH). Free Ca^{2+} was adjusted by altering the ratio of CaK_2EGTA/K_2EGTA, keeping the total EGTA at 4 mM. The composition of these solutions was calculated as described by Arner (1983).

The muscle fiber preparations were electrically stimulated using a Grass S44 stimulator (Quincy, MA), via platinum electrodes placed close to the muscle fiber in the two different mechanical setups described below. To activate the muscles maximally we used stimulation at 50 Hz and adjusted stimulus duration and voltage to obtain maximal force responses. We consider the responses to be mainly direct activation of the cells, since the responses could not be abolished by TTX. We cannot, however, exclude that a small nervous component contributes to the responses.

Instrumentation for fura-2 measurements in muscle fibers

Silk thread was tied around both ends of the muscle strip and it was then pulled into a perfusion cuvette similar to that described by Güth and Wojciechowski (1986). The quartz cuvette (length 10 mm, inner diameter 1 mm) was mounted in a temperature-controlled copper block. The silk thread at one end of the muscle was attached to an AME force transducer (SensoNor a.s., Horten, Norway) and that at the other end to a hook on a micrometer screw for length adjustment. Perfusion of fresh solution could be performed through an opening at one end of the cuvette. The sample was illuminated through an opening in the side of the copper block, and the emitted light was recorded from above the cuvette at a 90° angle relative to the excitation light. Thin platinum stimulation electrodes were placed along the muscle fiber preparation without disturbing the light path of the excitation and emission light.

A mercury light source (HBO 100W lamp using a lamp house and starter from Nikon, Tokyo, Japan) was used for excitation of fluorescence. The light was focused (focusing assembly model 77800, Oriel, Stratford, CT) on a liquid light guide (model 77556, Oriel) collimated on the filter wheel. The light from the filter wheel was focused on a second liquid light guide transferring the light to the muscle in the cuvette. Fused silica collimating beam probes (model 77646, Oriel) were used on the light guides on both sides of the wheel and in front of the muscle. The filter wheel (thickness 5 mm, diameter 100 mm) was made of aluminum with stainless steel ball bearings. The wheel was driven via a rubber belt by a DC-motor (Interelectric AG, Sachelsen, Germany). Six filters with diameter 13 mm (Ditric Optics, Hudson, MA), and wavelength alternating 340 nm (half-width 12 nm) and 380 nm (half-width 11 nm) were mounted in the filter wheel. The speed of the wheel was stable within 1% during the measurement intervals. Filters could be changed within 1 ms. In the present experiments the wheel was rotated at 16.7 Hz (one filter change per 10 ms). Two types of trigger pulses were generated by the filter wheel assembly (one pulse between each filter and one pulse each turn of the wheel).

Emitted light was recorded at 90° relative to the path of the excitation light through a Nikon SMZ-2T microscope. This binocular stereo microscope was used during mounting of the preparation. Measurements were then made through the camera mount, a Nikon diaphragm, and a shutter (PFX, Nikon) equipped with an eye piece using a Hamamatsu R374 photomultiplier. The slits in the diaphragm were adjusted so that the

muscle in the cuvette was just visible. A 500-nm (half-width 40 nm, diameter 24.5 mm, Ditric Optics) filter was mounted between the shutter and the photomultiplier. The signal from the photomultiplier, after applying current to the voltage converter (Hamamatsu C1053) was analyzed by analog integration. Because the diameter of the filters was about the same size as the diameter of the collimated light beam, the light intensity varied as the filter passed through the beam of light. To increase the accuracy of the measurements the emitted light was integrated before sampling. The trigger pulses from the filter wheel were adjusted to occur between the filters when light intensity was zero. These pulses were used to reset the integrator. Ten microseconds before resetting a sample was taken by an AD converter (Analog Devices RTI-800F). At the same time the force signal and a record of the filter number were stored. A computer program performed background subtraction and the calculation of the ratio between light intensities at 340 and 380 nm. The light signals recorded with shutters open without excitation light were used for background subtraction. An adequate time resolution was obtained in the present experiments by averaging the data over one turn of the wheel giving a sampling frequency of force and 340/380 ratio of 16.7 Hz.

Calibration of the instrument using fura-2 salt gave an apparent K_d of 0.19 μM at 22°C, which is similar to values given in the literature (Grynkiewicz et al., 1985). In muscle fiber experiments the muscles were loaded with fura-2 as described above and mounted in the cuvette. The muscle fiber preparation was stimulated at regular intervals and 3–5 contractions with measurements were made on each preparation.

Time-resolved spectroscopy

Time-resolved spectroscopy of free fura-2 and muscle fibers was performed at a beam-line for time-resolved spectroscopy at the MAX synchrotron light facility in Lund, Sweden (Rigler et al., 1987). This storage ring is a dedicated light source with properties suitable for time-resolved fluorescence spectroscopy and can give the light pulses (duration ~50 ps) with a repetition frequency of 10 MHz (single bunch mode) or 20 MHz (dual bunch mode). In the experimental setup at the beam line, synchrotron light is passed through an Acton Czerny-Turner grating monochromator for selection of excitation wavelength. Emitted light is recorded with a Hamamatsu (R1564U) microchannel plate for time-correlated single photon counting using a Nuclear Data pulse-height analyzer. Measurements were made of the dark current, the light pulse, and two polarized components of the emitted light [the magic angle (I_m) and the perpendicular angle (I_p)]. Time-resolved anisotropy was calculated from the time-resolved I_m and I_p measurements. An advantage of using synchrotron light is the ability to select excitation wave length. In the experiments on fura-2 fluorescence excitation was performed at 360 nm and the emitted light was passed through a 525-nm glass filter. Measurements were made at each component 20 times for 20 s each. Measurements were made in a quartz cuvette using 2 μM fura salt in the TES-buffer described above at two free [Ca^{2+}], 10^{-9}, and $10^{-4.5}$ M. To examine whether fura could be immobilized by protein, measurements were also made in the presence of creatine kinase (25 mg/ml). In measurements on muscle loaded with fura, rectococcygeus muscle fibers were treated by fura-2/AM as described above and mounted in the cuvette at an angle of 45° relative to the excitation and emission light, with the long axis perpendicular to the polarization of the excitation light.

Flash photolysis of diazo-2

Flash photolysis experiments were performed using a high-pressure xenon flash lamp system (Arner et al., 1987) giving light flashes with a duration of <5 ms. A UG11 filter was used to eliminate longer wavelengths. Aluminum foil was wrapped around both ends of the preparation and the muscle was then attached via holes made in the aluminum between a fixed hook and the extended arm of an AME force transducer. The muscle was held horizontally in 0.5 ml perspex baths, which were replaced at regular intervals for solution exchange. The muscle could be stimulated by means of two platinum electrodes mounted in parallel with the muscle. Before photolysis the muscle was transferred to a 100-μl bath equipped with stimulation electrodes. The muscle was illuminated, when in solution, through a quartz window in the cuvette. No difference could be noted between contractions elicited in the 0.5-ml baths and in the cuvette for photolysis experiments.

The muscle fibers were loaded with diazo-2 as described above and one photolysis experiment was performed on each preparation. After mounting in the apparatus, each fiber preparation was electrically stimulated at 5-min intervals. When the contractions became reproducible with peak forces differing <5%, a control response was recorded. During the subsequent contraction the muscle was illuminated at a specific point in time during the contraction. After this contraction a final control contraction was elicited.

The force transients were stored on magnetic tape (Racal, Thermionic store IV, Hythe, Southampton, UK) and digitized at 333 Hz using an Analog-Devices RTI-800F board in an XT-personal computer.

Statistics

All data are given as mean ± SEM. Comparisons between groups were made using the Student's *t*-test.

RESULTS

Intracellular calcium transients

Strong fluorescence signals were observed after 2 h of loading with fura-2/AM at room temperature showing that fura-2 was accumulated in the rectococcygeus muscle preparations. The background from the internal fluorescence and scattering was very low in these muscles. In fiber preparations mounted without prior treatment with fura-2/AM the fluorescence was ~5% of that in the treated muscles, and no alterations in the 340 and 380 signals were seen when the muscles were activated. The time to develop half-maximal tension after the start of stimulation was unaltered and the time to relax from peak to half-maximal tension was slightly slower (by ~14%, $P < 0.05$) compared to corresponding values during contractions in nontreated muscles studied in the same mechanical setup (*open* and *hatched bars* in Fig. 1 *b*). We do not have an explanation for the slowing of relaxation effect, but consider it an unspecific effect of possibly the fura loading procedure. During activation, the fluorescence intensity at 340 nm excitation increased and that at 380 nm decreased. Fig. 1 *a* shows the calculated ratio between the fluorescence intensities (340/380 ratio) during an electrically induced contraction in a rectococcygeus muscle preparation. Stimulation is associated with a rapid increase in the 340/380 ratio, reflecting an increase in intracellular [Ca^{2+}]. The rise in [Ca^{2+}] precedes force development. The delay between onset of stimulation and force was 225 ± 24 ms ($n = 5$). On average, the apparent half-time for the rise in [Ca^{2+}] after activation was almost fourfold shorter than the rise in force (*filled bars* in Fig. 1 *b*). Half-maximal Ca^{2+} was reached 1.9 ± 0.2 s ($n = 14$) before the half-maximal force. After cessation of stimulation [Ca^{2+}] starts to fall almost immediately, whereas force development continues for a brief period and the muscle then relaxes. The half-time for the fall in [Ca^{2+}] was

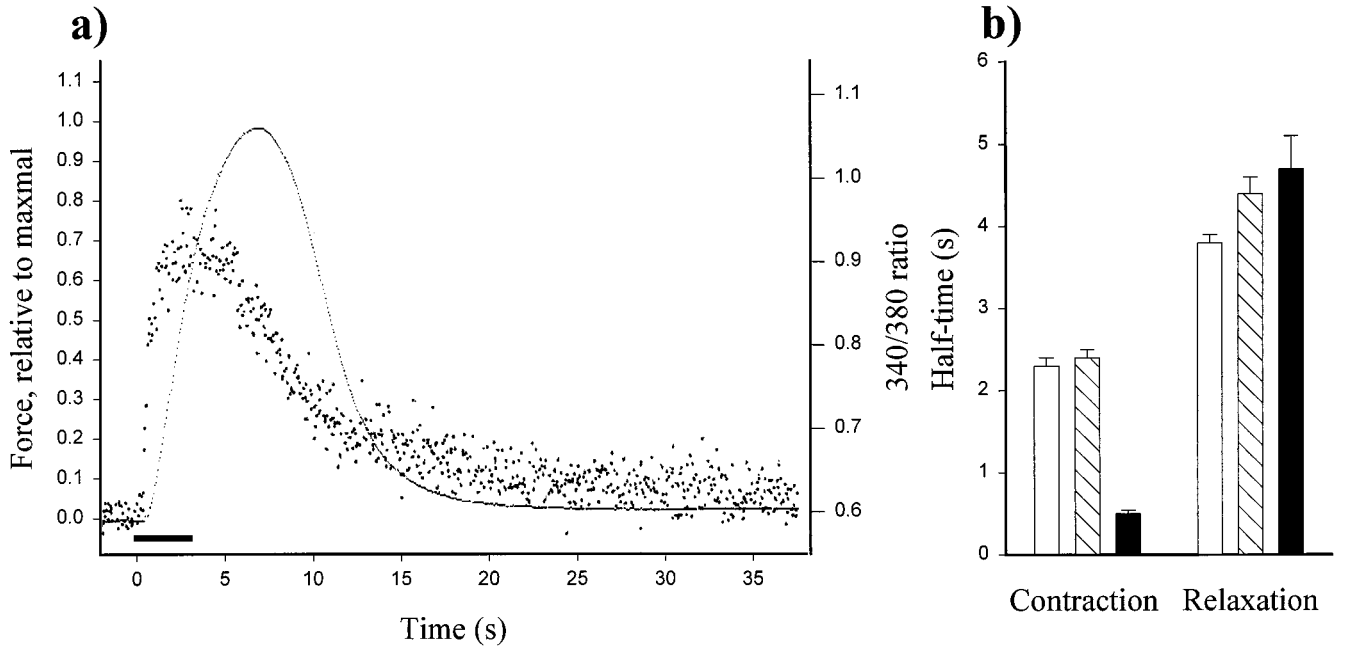

FIGURE 1 Intracellular calcium and force during an electrically induced contraction of a rectococcygeus muscle preparation. (*a*) Force (*continuous line*) and the fura-2 signal, expressed as the ratio between the fluorescence intensities at 340 and 380 nm (*dots*). Stimulation indicated by bar below the record. (*b*) Time to reach half-maximal response for force (*open bars* show untreated control fibers, $n = 4$, and *hatched bars* show the fura-2-treated fibers, $n = 14$) during the contraction and relaxation phases. The half-times for Ca^{2+} rise and Ca^{2+} fall (fura-2 signal), during contraction and relaxation, respectively, are shown with filled bars.

similar to the half-time for the fall in force (Fig. 1 *b*), but the fall in Ca^{2+} preceded the fall in force by 3.5 ± 0.3 s ($n = 14$).

Time-resolved spectroscopy of fura-2

Fig. 2 (*top*) shows the measured synchrotron light pulse at 360 nm. The bottom panel shows the corresponding fluorescence decay of free fura-2 (2 μM) at low (pCa = $-\log[Ca^{2+}]$ 9) and high (pCa 4.5) [Ca^{2+}]. The fluorescence decay of fura-2 was dependent on the free Ca^{2+} concentration; increased concentration gave a slower decay. By using deconvolution and curve fitting we found that the data were best described by two exponential decays at low Ca^{2+} (time constants 0.5 and 1.5 ns, with respective relative amplitudes of 0.4 and 0.6, $x^2 = 2.28$) and by one exponential decay at high Ca^{2+} (time constant 1.9 ns; $x^2 = 1.3$).

The time-resolved anisotropy of free fura-2 (at pCa 9) is shown in the top panel of Fig. 3. The anisotropy decays rapidly toward zero within a few nanoseconds. Curve fitting gave a rotational correlation time of 0.33 ns, which is characteristic for fast free rotational diffusion. The bottom panel shows the effects of addition of creatine kinase (25 mg/ml). The anisotropy shows an initial fast component, most likely due to scattering, but does not decay toward zero within the observation time. This high limiting anisotropy shows that a large fraction of the fura-2 molecules are immobilized by the protein.

Fig. 4 (*top*) shows time-resolved fluorescence and anisotropy decay from a relaxed rectococcygeus muscle fiber loaded with fura-2 as described above. Compared with the data from free fura-2 the fluorescence lifetimes were slightly longer in the muscle fiber, suggesting changes in the chemical environment of the fluorescent probe. The time-resolved anisotropy from the fiber had an initial fast component followed by a decay to zero. These data suggest that the fluorescent probe has a high degree of rotational freedom when loaded into the muscle cells.

Mechanical effects of photolysis of diazo-2

No influence on the mechanical behavior of the rectococcygeus fibers could be noted after treatment with diazo-2/AM. The times to develop half-maximal tension after the start of stimulation (2.2 ± 0.1 s, $n = 15$) and to relax from peak to half-maximal tension (6.6 ± 0.6 s, $n = 15$) were similar to the corresponding values during contractions in nontreated muscles studied in the same mechanical setup (contraction: 2.5 ± 0.1 s, $n = 15$; relaxation: 7.5 ± 0.9 s, $n = 15$). These values also correspond well with data obtained in the fura-2 experiments described above (Fig. 1 *b*), although the rate of relaxation of both controls and diazo-treated muscles was slower in this series of experiments. In nontreated muscles, illumination did not influence the force responses when performed during the rising phase or during the relaxation, showing that the light pulse itself

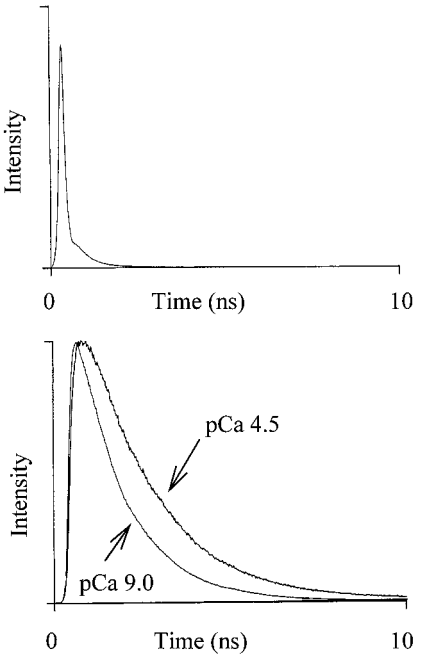

FIGURE 2 Time-resolved fluorescence of fura-2. The top panel shows the synchrotron light pulse at 360 nm. The bottom panel shows the corresponding fluorescence of fura-2 at pCa 9 and pCa 4.5. The maximal intensity on the *y* axis was 2.4×10^5 counts for the light pulse, and 7×10^4 and 1.6×10^4 counts for the recordings at low and high $[Ca^{2+}]$.

does not influence the contraction or relaxation processes. Fig. 5 shows an experiment where the muscle was illuminated during the rising phase of contraction when force had reached ~50% of its peak value. The force recording is superimposed on that of the preceding control contraction in the same fiber. Note that the force traces superimpose

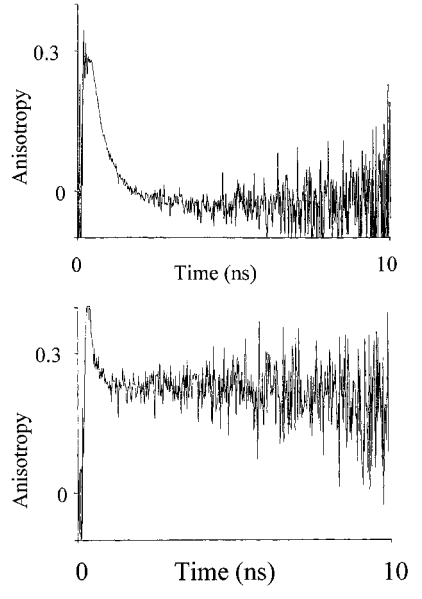

FIGURE 3 Time-resolved anisotropy of fura-2 in solution. Measurements were made in pCa 9.0. In the experiment shown in the bottom panel creatine kinase (25 mg/ml) was added.

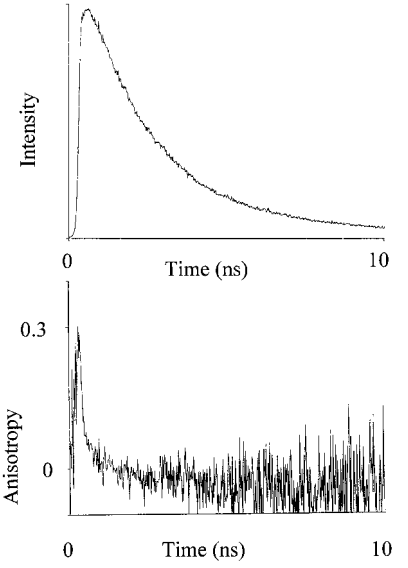

FIGURE 4 Time-resolved fluorescence (*top*, maximal intensity was 4.5×10^3 counts) and anisotropy (*bottom*) of a relaxed rectococcygeus muscle loaded with fura-2.

completely up to the time for the light flash. The illumination resulted in an immediate change in the rate of force development (the tangent at the time point for the flash was reduced to ~50%). The maximum force developed after the flash was reduced to ~90% of that during the control contraction. A total of eight flash experiments were performed at different relative force levels during the rising phase (range 25–80% of the control response). Only one photolysis experiment was performed on each diazo-2/AM-treated fiber preparation. The rate of force development was evaluated as the tangent drawn to the force record immediately before and after the light flash. On average, the rate decreased to 69 ± 8% ($n = 8$) after the light flash. The largest effects were noted at ~50% relative force and the effect was smaller at the highest force levels.

The peak force reached during the contraction when the muscles had been illuminated during the rising phase was on average 86 ± 3% ($n = 8$) of the control response. This inhibition of peak force was not due to variability between contractions, because in contractions where the muscles were illuminated during the falling phase peak force was 100 ± 2.5% ($n = 6$) of the control response. The control contraction at the end of the experiment after a contraction where the diazo-2/AM-treated muscle had been had illuminated was 81 ± 3% ($n = 12$) of the control response, showing that the photolysis of diazo-2 gave a sustained reduction of peak force.

When photolysis was performed during the falling phase of the contractions no effects could be noted on the rate of tension decline. Fig. 6 shows representative force traces from two fiber preparations where photolysis was performed during the relaxation at a force level, relative to peak force of ~80 (*top*) and 40% (*bottom*). The peak reached during these contractions before the light flash was close to

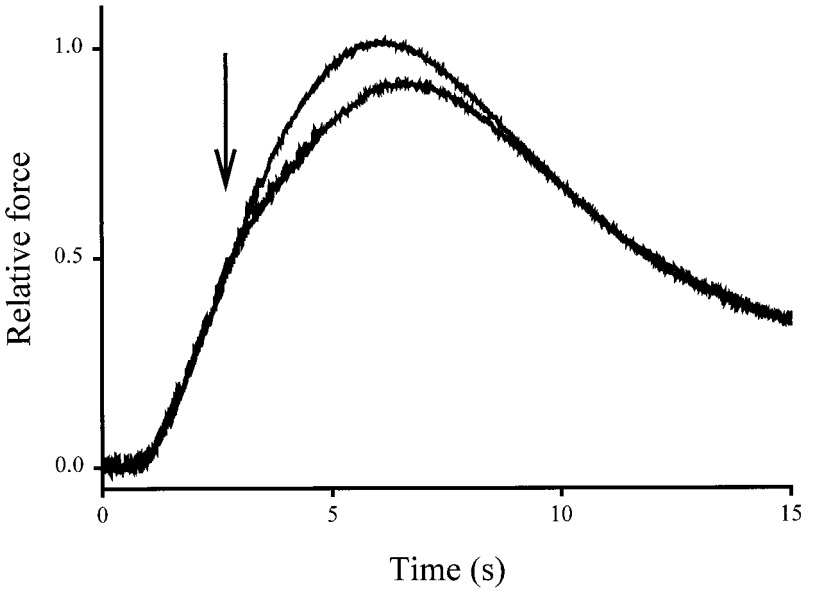

FIGURE 5 Effects of a light flash (*arrow*) during the rising phase of contraction in a rectococcygeus muscle fiber preparation treated with diazo-2/AM. The force trace from the contraction with illumination is superimposed on that of the preceding control contraction.

that of the control contractions, as discussed above, and the peak of the subsequent contraction was inhibited, showing that photolysis of diazo-2 had occurred. In total, eight flash photolysis experiments were performed at different relative force values during relaxation (range 90–40%). In neither of these could any effect on the rate of relaxation be observed.

DISCUSSION

The rectococcygeus muscle is a comparatively fast smooth muscle (Malmqvist and Arner, 1991) with highly reproducible electrically induced contractions. We show that these contractions are associated with a transient increase in intracellular [Ca^{2+}]. The increase in [Ca^{2+}] clearly precedes force development and the previously reported changes in myosin structure associated with the contractions (Arner et al., 1989). Little is known about the fluorescence properties of fura-2 in smooth muscle fibers, and a change in the mobility or fluorescence of the probe could have effects on the interpretation of Ca^{2+} transients. The fluorescence measurements on free fura-2 gave fluorescence lifetimes and a rotational correlation time similar to those described previously by Keating and Wensel (1991). In accordance with their study, we also found that high concentrations of protein (creatine kinase) affected both fluorescence lifetimes and the anisotropy decay, suggesting that protein binding influences the fluorescence emission and the rotational mobility of the fluorophore. The situation in the smooth muscle fiber preparation appears to be complex. The fluorescence decay revealed longer lifetimes than those observed in free fura-2; the time constants observed in the relaxed muscle were slightly higher than those of free fura-2 at high Ca^{2+}. In frog striated muscle fibers the fluorescence properties of the steady-state fluorescence properties of intracellular fura-2 were found to differ from those determined in vitro (Konishi et al., 1988). This was interpreted to be a result of binding of fura-2 to intracellular macromolecules. On the basis of time-resolved fluorescence measurements (Keating and Wensel, 1991) suggested that a significant, but variable, fraction of fura was immobilized in blood cells. In contrast, Williams et al. (1985) found no difference in steady-state anisotropy between fura-2 in vitro and when loaded in the cytoplasm of amphibian smooth muscle cells. Our finding that fluorescence lifetimes are altered suggests a change in the chemical environment of the fura-2 probe when loaded into the rectococcygeus muscle fibers. However, no significant limiting anisotropy could be found, suggesting that the mobility of the probe was not influenced to a large extent. The changes in fluorescence lifetimes upon loading suggest, however, that it is not straightforward to use the in vitro calibration of the fura-2 signal to transform the ratio determinations from living cells to intracellular Ca^{2+} concentration values. Because we were interested in the time course Ca^{2+} transients, rather than in absolute concentrations, we have used the 340/380 ratio.

Activation of the rectococcygeus muscle fiber preparations resulted in a rapid increase in intracellular [Ca^{2+}], followed by force development at a slower rate. The Ca^{2+} influx or release appears to be directly controlled by the electrical activation inasmuch as [Ca^{2+}] starts to increase rapidly after onset of stimulation and falls almost immediately after cessation of stimulation. The apparent rate constant for the rise in force calculated from half-time for force development was $\sim$0.3 s^{-1}. When contractions are elicited in chemically skinned rectococcygeus muscles, fully activated by irreversible thiophosphorylation of the regulatory light chains on myosin, force development occurred at an apparent rate of 1 s^{-1} (Malmqvist and Arner, 1991). These results, together with the Ca^{2+} measurements, suggest that the force development in the electrically induced contractions is not rate-limited by the rate of Ca^{2+} influx or by the

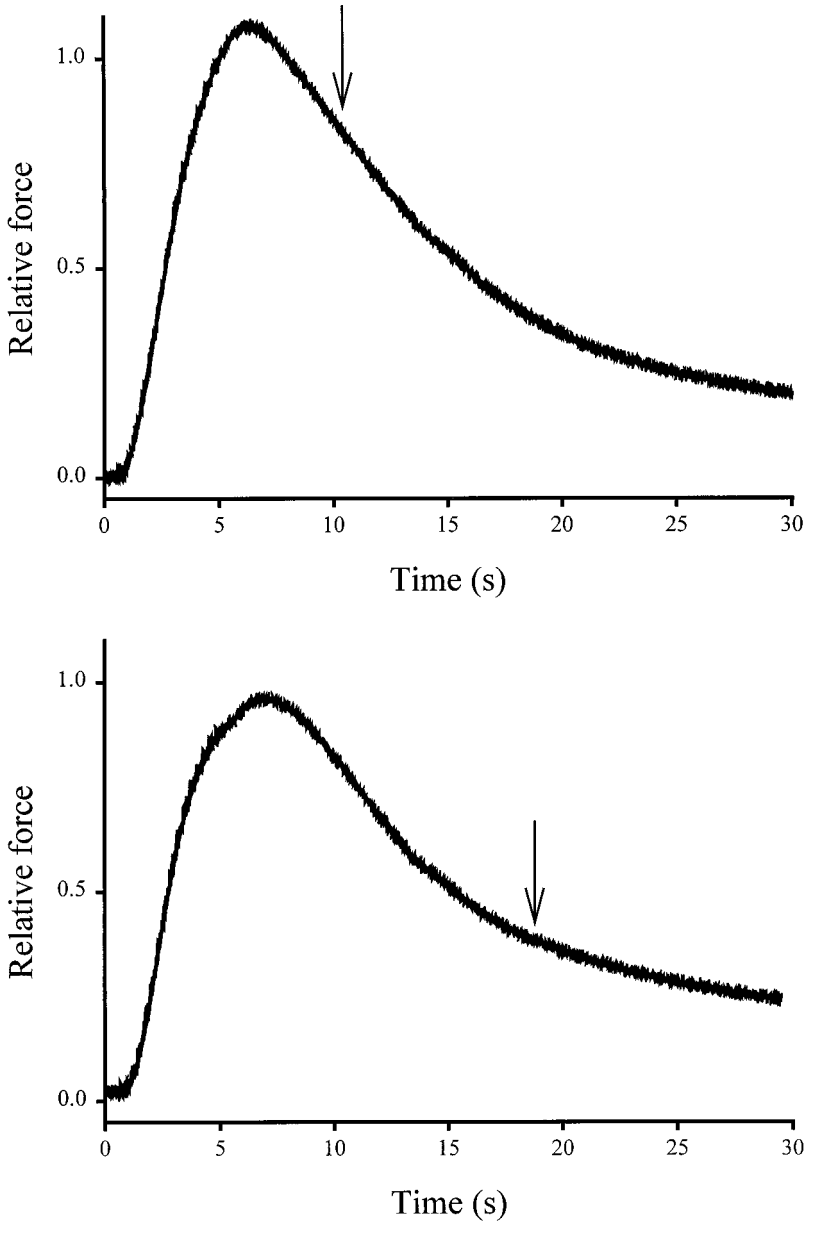

FIGURE 6 Effects of a light flash (*arrows*) during the falling phase of contraction in two rectococcygeus muscle fiber preparations treated with diazo-2/AM.

force development of a maximally activated cross-bridge system. It seems possible that a slower activation step after the Ca^{2+}-increase is responsible for the delay between the $[Ca^{2+}]$ increase and force development. In electrically stimulated intact muscle, Kamm and Stull (1986) have shown that light chain phosphorylation and muscle stiffness are closely coupled, whereas there is a delay between these parameters and force. Fischer and Pfitzer (1989) found a close correlation between phosphorylation and force during the initial phase of tension development in carbachol and electrically activated chicken gizzard fibers. Studies using chemically skinned fibers show that the rate of force development can be influenced by the myosin light chain kinase/phosphatase activities (Horiuti et al., 1989). In skinned muscle a delay between phosphorylation and force development at optimal $[Ca^{2+}]$ has been reported (Kühn et al., 1990) and it is possible that other Ca^{2+} regulatory mechanisms or reactions in partially activated cross-bridges are influencing force development. However, by using permeabilized skinned preparations and activation with photolysis of caged-Ca^{2+}, shorter delays between the rise in phosphorylation or $[Ca^{2+}]$ and force have been reported (Zimmermann et al., 1995). Thus, a likely rate-limiting process for the initial rate of tension development in the electrically stimulated rectococcygeus muscle is the rate of myosin phosphorylation. Interestingly, force development continues after $[Ca^{2+}]$ starts to decline (Fig. 1), which could reflect that $[Ca^{2+}]$ has reached a saturating level or that a secondary process causes further force development. A similar continuation of force development after the peak in light chain phosphorylation has been observed in phasic contractions in chicken gizzard fibers (Fischer and Pfitzer, 1989).

An analysis of the relationship between force and phosphorylation or [Ca^{2+}] during non-steady-state conditions should be regarded with caution, but the continuing force development when the [Ca^{2+}] and phosphorylation have started to decline could reflect the recruitment of nonphosphorylated cross-bridges, possibly via regulation by phosphorylation-independent regulatory processes, such as cooperative attachment (Himpens et al., 1988; Somlyo et al., 1988).

A rapid decrease in the rate of tension development was induced by photolysis of the caged [Ca^{2+}] chelator, diazo-2. The effects of diazo-2 photolysis were observed during the rising phase of the contraction when [Ca^{2+}] was at its maximum and the effect was most likely due to a reduction of free [Ca^{2+}]. We could not, however, determine the extent of diazo-2 loading in the fibers nor the degree of photolysis, but the released calcium chelator was obviously not sufficient to reduce Ca^{2+} to the relaxed levels, because the muscle did not relax in response to photolysis of diazo-2. Inasmuch as the rate of Ca^{2+} increase was very fast (half-time 0.5 s for Ca^{2+} rise vs. 2.5 s for force) the force increase appears not to be limited by the rate of calcium increase. However, the diazo-2 data show that during the rising phase of the contraction, force development is coupled to the free Ca^{2+} level, or more likely the level of phosphorylation (Kamm and Stull, 1986; Fischer and Pfitzer, 1989).

The relaxation of the muscle was preceded by a fall in Ca^{2+}. During the relaxation, force was maintained at low levels of Ca^{2+}. This is similar to steady-state measurements on depolarized vascular tissue where force can be maintained at low [Ca^{2+}] and phosphorylation levels. (cf. Rembold and Murphy, 1986). It has been suggested that the rate-limiting reaction for relaxation is the removal of calcium in arterial smooth muscle (Rembold, 1991). During the relaxation of the rectococcygeus muscle we have a situation where force is high and [Ca^{2+}] low, meaning that either force is maintained by a Ca^{2+}-independent system or by a system that has a high Ca^{2+} sensitivity. Our flash photolysis experiments show that a fall in [Ca^{2+}] does not increase the rate of relaxation in the rectococcygeus muscles. The lack of effects of diazo-2 photolysis during relaxation was not due to insufficient release of chelator because the rate of tension development could be influenced by photolysis during the rising phase of the contraction when Ca^{2+} was high. Considering the dissociation constants for diazo-2 before and after photolysis it is further expected that the relative decrease of free [Ca^{2+}] is higher at low [Ca^{2+}] concentrations. Thus, the relaxation is not rate-limited by Ca^{2+} removal and force maintenance during relaxation is not due to a Ca^{2+}-dependent regulatory system with a high sensitivity to Ca^{2+}. The effect of Ca^{2+} on force thus appears to be switchlike, possibly due to a very steep Ca^{2+} concentration dependence of the MLCK activity. Force starts to increase at a Ca^{2+} level ~25% of maximal (Fig. 1) showing that the MLCK activity initially responds to low Ca^{2+} and is dependent on Ca^{2+} concentration. If the Ca^{2+} dependence of MLCK does not change with time during contraction, then a substantial MLCK activity would be expected to be present also during the relaxation phase. Because release of the calcium chelator did not reduce force during relaxation, MLCK activity is not responsible for activation of cross-bridge interaction during the relaxation phase. This suggests that the MLCK becomes desensitized to Ca^{2+} and is fully turned off during the relaxation. A possible mechanism could be a phosphorylation of the MLCK by the Ca^{2+}/calmodulin-dependent protein kinase II, similar to that found during contractions in myometrial smooth muscle (Word et al., 1994). The results also exclude a secondary regulatory system controlling force at low Ca^{2+} during relaxation. The present results show that, whereas initial force development during electrical stimulation is tightly coupled to the Ca^{2+} concentration, the relaxation is independent of free [Ca^{2+}] and most likely determined by the rate of detachment of dephosphorylated cross-bridges from tension-generating states or by the rate of deactivation of phosphorylation-independent regulatory systems. Such regulatory systems could be cooperative mechanisms on the thin filaments, possibly enhanced by MgADP accumulation (Khromov et al., 1995) during the phasic contraction, or other thin filament-associated regulatory systems.

The authors thank J. Roslund for help during the experiments at the MAX-synchrotron light facility.

This study was supported by Swedish Medical Research Council Grants 04x-8268 and 04x-12584, the Crafoord Foundation, and Kungliga Fysiografiska Sällskapet, Lund.

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