Force Regulation by Ca^{2+} in Skinned Single Cardiac Myocytes of Frog

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ABSTRACT Atrial and ventricular myocytes 200 to 300 μm long containing one to five myofibrils are isolated from frog hearts. After a cell is caught and held between two suction micropipettes the surface membrane is destroyed by briefly jetting relaxing solution containing 0.05% Triton X-100 on it from a third micropipette. Jetting buffered Ca^{2+} from other pipettes produces sustained contractions that relax completely on cessation. The pCa/force relationship is determined at 20°C by perfusing a closely spaced sequence of pCa concentrations (pCa = $-\log[Ca^{2+}]$) past the skinned myocyte. At each step in the pCa series quick release of the myocyte length defines the tension baseline and quick restretch allows the kinetics of the return to steady tension to be observed. The pCa/force data fit to the Hill equation for atrial and ventricular myocytes yield, respectively, a pK (curve midpoint) of 5.86 $\pm$ 0.03 (mean $\pm$ SE.; $n = 7$) and 5.87 $\pm$ 0.02 ($n = 18$) and an n_H (slope) of 4.3 $\pm$ 0.34 and 5.1 $\pm$ 0.35. These slopes are about double those reported previously, suggesting that the cooperativity of Ca^{2+} activation in frog cardiac myofibrils is as strong as in fast skeletal muscle. The shape of the pCa/force relationship differs from that usually reported for skeletal muscle in that it closely follows the ideal fitted Hill plot with a single slope while that of skeletal muscle appears steeper in the lower than in the upper half. The rate of tension redevelopment following release restretch protocol increases with Ca^{2+} >10-fold and continues to rise after Ca^{2+} activated tension saturates. This finding provides support for a strong kinetic mechanism of force regulation by Ca^{2+} in frog cardiac muscle, at variance with previous reports on mammalian heart muscle. The maximum rate of tension redevelopment following restretch is ~twofold faster for atrial than for ventricular myocytes, in accord with the idea that the intrinsic speed of the contractile proteins is faster in atrial than in ventricular myocardium.

INTRODUCTION

Contraction in mammalian skeletal and cardiac muscle in vivo is regulated primarily by calcium ions (Ca^{2+}) binding to troponin C on the thin filament. The thin filament is built up of two actin and two tropomyosin strands with troponins T, I, and C attached at regular intervals. Troponin C (TnC) has four Ca^{2+} binding sites, two of high affinity, that are important in keeping TnC bound to the thin filament (Zot and Potter, 1982) and two sites of lower affinity (one for cardiac TnC) that regulate contractile activation (Potter and Gergely, 1975). The role of Ca^{2+} in regulation of contraction is most simply demonstrated with permeabilized (skinned) fibers in which the Ca^{2+} and $MgATP^{2-}$ around the myofilaments are buffered by the bathing solution.

Initially, pCa/tension data fit to the Hill equation were found to have a Hill coefficient, n_H, of 1 to 2, a range explicable by assuming that the two low affinity Ca^{2+} binding sites on troponin C bound Ca^{2+} with some degree of cooperativity. However, with technical improvements and the introduction of the "serial dilution method" of generating a dense pCa series in the muscle chamber, the n_H of rabbit psoas is found to be 5 or greater (Brandt et al., 1980), implying that there is a high degree of cooperativity between Ca^{2+} binding sites involving at least several TnC's along the thin filament. Recent studies from one of these laboratories (Brandt et al., 1984a,b, 1987, 1990; see also Fraser and Marston, 1995) suggest that the cooperativity probably encompasses all the proteins of the thin filament, which switches "off" and "on" as a unit.

Skinned multicellular preparations of cardiac muscle are commonly reported to have an n_H of 1 to 3 (e.g., Brandt and Hibberd, 1976; Best et al., 1977; Kerrick et al., 1980; Hibberd and Jewell, 1982; Stephenson and Wendt, 1986; Gulati et al., 1988; Morimoto and Ohtsuki, 1994) though some higher values have occasionally been reported (Kentish et al., 1986). Relatively small n_H values have also been found in single cell preparations of cardiac muscle (Fabiato, 1982; Sweitzer and Moss, 1990; Araujo and Walker, 1994; Strang et al., 1994) as well as in single myofibrils (Linke et al., 1994). Many workers expect the Hill coefficient of cardiac muscle to be smaller than that of fast skeletal muscle because cardiac TnC has only one Ca^{2+} regulatory binding site, compared to two for skeletal muscle (Moss et al., 1986; Brandt et al., 1987; for review see Schiaffino and Reggiani, 1996). Here we reexamine this issue and introduce several technical enhancements to the study of heart muscle myofibrils.

While studies on single fibers reduce the diffusion delays and artifacts common to studies on strips of muscle, even these are large enough to have problems with diffusion. Ideally one would like to study one sarcomere of one myofibril held without crushing, gluing, or chemically altering the proteins at the point of attachment to the apparatus. A close approach to this ideal is a preparation derived from intact atrial and ventricular frog myocytes (Cecchi et

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al., 1992; Colomo et al., 1997b) that contain one to five myofibrils. These are held in suction pipettes without mechanical or chemical alteration of their ends. After the myocytes are skinned the remaining myofibrils are close in size to the isolated myofibrils studied by biochemical methods, but this preparation has the added advantage in that the myofibrils are kept isometric. We have adapted the automated serial dilution technique to these minute skinned preparations and determined their pCa/force relation. In addition, some kinetic properties are also measured. At each steady tension following a pCa change the length of the myocytes is rapidly released and the tension baseline determined. Then, after a pause, they are restretched and the time course of tension redevelopment and reequilibration observed.

METHODS

The experimental chamber

The experimental chamber shown in Fig. 1 is used to perfuse myocytes with a closely spaced series of pCa solutions in the range 8 to 4.75. The chamber, machined out of an aluminum disk, contains two wells (L and R in Fig. 1) ~1 cm on each side with a 1-cm platform between them. The platform is hollow and a glass coverslip is glued over the hole through the chamber; illumination is directed down through the solution on the platform into an inverted Nikon microscope objective located below the coverslip. Two suction micropipettes are shown terminating just above the coverslip on the platform. The preparation ends are held in the tips of these pipettes. The aluminum disk has channels in it through which temperature-regulated water is circulated; this keeps the solution in the chamber and on the platform at a constant temperature.

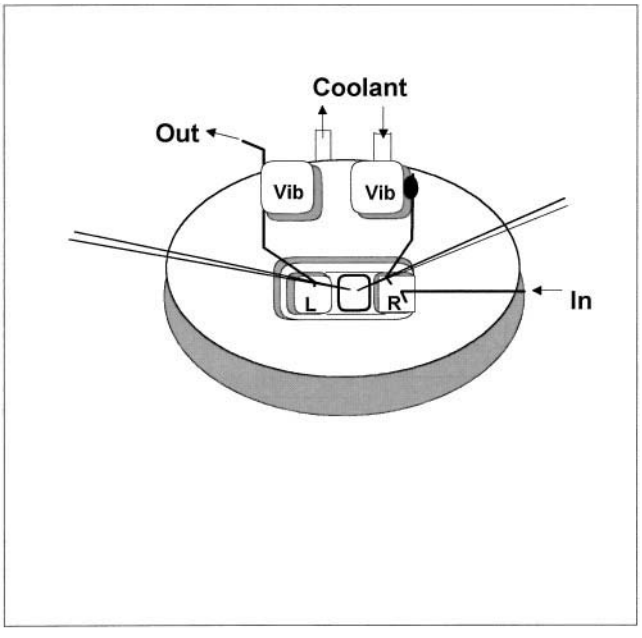

FIGURE 1 Experimental chamber. Both left (L) and right (R) wells are filled with relaxing solution until the solution in the center platform is 1 mm deep and each contains ~1 ml. The myocyte is held on the center platform between the two glass suction pipettes shown projecting over the platform. Injection of 88 μl pCa 4.75 solution through the input tube (at the "in" arrow) causes the same volume to flow across the central platform into L. Vibrating SS tubes (Vib = motor) stir both wells; that in L has a small hole in its side through which a vacuum can suck the solution down to a fixed level. The left end is connected through an electronic valve to the vacuum source. The tips of the SS tubes in L and R are flattened to seal them and improve agitation of the solution.

Initially both L and R are filled with pCa 8 relaxing solution until a 1-mm depth of solution covers the middle platform. L and R each contain ~1 ml of solution. Small volumes of pCa 4.75 activating solution are injected at fixed intervals through the input tube inserted into R ("in" arrow in Fig. 1) by a computer-controlled, stepping motor-driven, precision pump (Fluid Metering Inc., Oyster Bay, NY) (Brandt et al., 1980, 1984a). Solution is removed through the output tube inserted into L ("out" arrow in Fig. 1) by a vacuum source connected to the output tube through an electronic valve.

A vibrating stirrer made from stainless steel tube is immersed in each well and its end is crushed flat to seal it and improve agitation of the solution. That in L has a small hole in its side just above the seal so that when the vacuum is applied it sucks the solution down to a fixed level. Vibration of the tube breaks surface tension between the solution in L and the orifice so that aspiration of solution stops at a constant level and sets the volume of solution in L. At each pCa 4.75 addition the vacuum valve is opened for 3 s.

Injection of 88 μl of pCa 4.75 solution through the input tube into R forces the same volume to flow across the platform and into L (see "pCa/force relationship" below).

Experimental apparatus

The experimental apparatus is centered about an inverted microscope (Nikon Diaphot, Japan) placed on an air suspension table and equipped with bright field and phase contrast optics. Images of the preparations at rest and during the skinning procedure are projected onto a CCD camera and recorded on video tape. The present working conditions of the force transducer do not allow us to take video images of the preparation during tension recording. The force transducer and the length control motor are the same as described previously to measure and control force and length of intact frog cardiac myocytes (Colomo et al., 1994). Force and length signals are recorded by a Nicolet digital oscilloscope (model Pro20, Madison, WI) and a chart recorder (Rectigraph 8K, Nec San-ei, Japan). The force signal is also recorded by an IBM PS2 AT computer.

Myocyte preparation and mounting

Intact atrial and ventricular myocytes are enzymatically isolated from the frog heart as reported previously (Cecchi et al., 1992) and resuspended in pCa 8 relaxing solution. A small volume of the cell suspension is placed on the platform of the chamber filled with pCa 8 solution (Fig. 2 *A*). The myocytes are allowed to settle onto the glass platform, then one is visually selected and its tapered ends are sucked into glass micropipettes. The end segments of the holding pipettes are bent (Fig. 2 *B*) so that the part of the cell sucked into the pipette is 90° to most of the length of the cell. This bend greatly improves the retention of the myocytes in the pipettes. The pipette with the shorter bent segment (on the left in Fig. 2 *B*) is relatively stiff and is fixed to the lever arm of the length-control motor. The other pipette is held in a micromanipulator and doubles as a cantilever force probe of calibrated compliance. Force is measured by photoelectronically recording the deflection of the force probe from its resting position (Colomo et al., 1994). Force probes built for present experiments have a compliance of 1–5 nm nN^{-1} and a frequency response of 2–5 kHz in the experimental solution. During maximum force generation myocyte shortening due to force probe compliance is <3% of its slack length.

Sarcomere length and myocyte cross-sectional area

Resting sarcomere length, overall length, and width of frog atrial and ventricular myocytes are measured on video images taken through phase contrast optics. The total magnification is usually ×4000.

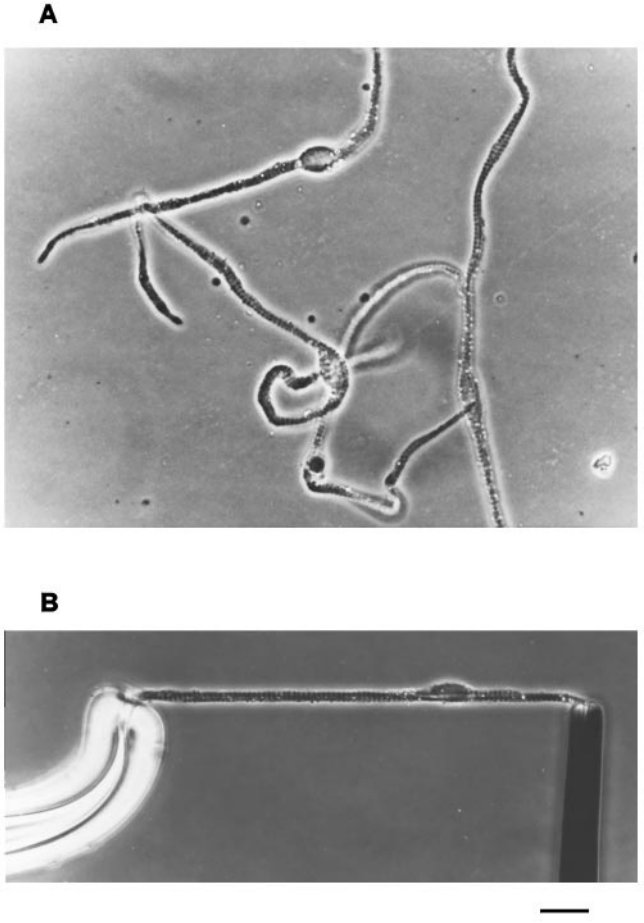

FIGURE 2 Photomicrographs of frog cardiac myocyte preparation. (*A*) Phase contrast image of a suspension of frog atrial myocytes on the central platform of the experimental chamber. Frog cardiac myocytes contain approximately one to five individual myofibrils. (*B*) Phase contrast image of a skinned atrial myocyte mounted between the two suction micropipettes. The suction micropipette connected to the motor lever arm (*left*) is bent to increase the efficacy of the attachment of the preparation. The cantilever force probe pipette tip (*right*) is blackened by a felt micropigment ink pen to increase the sensitivity of the force probe photosensor. Scale bar = 20 μm.

The striation spacing is determined by averaging the measurements of sequences of 10 sarcomeres displayed in the central region of video images. Sarcomere slack length is essentially the same in both types of myocytes averaging 2.07 ± 0.06 μm (mean ± SE.; n = 12) in the atrial myocytes and 2.09 ± 0.04 μm (n = 23) in the ventricular myocytes.

The width of both types of preparations are measured in the intact myocyte before skinning in relaxing solution at slack sarcomere length. As frog cardiac myocytes have a bulging central region that contains the nucleus and two spindle-shaped side regions (Fig. 2 *B*), widths vary significantly along the preparation. The average width of the side regions of the myocyte are used to calculate a rough estimate of the cross-sectional area of the cell, assuming it to be a right cylinder. Average values of cross-sectional area are significantly larger in ventricular myocytes (19.63 ± 2.00 μm^2) as compared to atrial myocytes (10.18 ± 1.54 μm^2).

Solutions

The solutions used to dissociate intact myocytes from the frog heart are described elsewhere (Cecchi et al., 1992). The standard pCa 8 "relaxing" solution contains (all are sodium salts in mM): 9.8 EGTA, 0.2 CaEGTA, 3 MgATP, 3 free ATP, and 10 MOPS. The pCa 4.75 activating solution contains 10 CaEGTA, 3 MgATP, 3 free ATP, and 10 MOPS. All solutions contain sodium propionate and Na_2SO_4 to adjust the final solution to an ionic strength of 0.130 and monovalent cation concentration of 90 mM. The concentrations of multivalent ionic species are calculated (Brandt et al., 1972) after solving the multiple equilibria of two metals (calcium, magnesium) and two ligands (EGTA and ATP), by using the following apparent association constants (log values at pH 7.00): CaEGTA 6.3, MgEGTA 1.6, CaATP 3.7, MgATP 4.1. Both experimental solutions are adjusted to pH 7.00 ± 0.01. The temperature of the chamber is maintained at 20.0 ± 0.1°C.

The reagent grade chemicals used in this study were purchased from the following sources: H_4EGTA, MOPS, Na_2H_2ATP from Sigma, St. Louis, MO; $CaCl_2$ (standard 1.0 M solution) from BDH, United Kingdom; $MgCO_3$, sodium propionate, propionic acid, from Merck, Darmstadt, Germany.

Skinning conditions

"Skinning solution" (pCa 8 relaxing solution containing 0.05% Triton X-100) is jetted for ~5 s at the mounted myocyte from a pressure pipette positioned close to the myocyte. The moment of skinning is usually visible as a sudden enhancement of the striation pattern and transient increase of the myocyte diameter. Long exposure to skinning solution or much higher concentrations of Triton X-100 result in the slow dispersal of the myofibrils. With the short duration of the jet and low detergent concentration used in this study the myofibrils are stable.

Ca^{2+} electrode

In some of the experiments the pCa of the solution on the platform of the experimental chamber was monitored by a small Ca^{2+} sensitive electrode (World Precision Instruments, Sarasota, FL). The electrode tip (diameter 1.0 mm) is immersed in the solution on the platform near the myocyte. Its output is connected to a custom electrode amplifier inserted between the chart recorder and the computer.

Computer system

An interface combining eight channels of A/D 12-bit resolution and 16 digital output lines (PCL-711S, Advantech Jamco, Sunnyvale, CA) provides communication between the IBM PS2 AT computer and the apparatus. The digital lines strobe the length driver logic and the Nicolet, command the solution pump, the suction pump and valve, the stirring motors, and event markers on the chart recorder. One analog line digitizes tension and another digitizes the Ca^{2+} electrode output. The experiment control program is written in Turbo Pascal.

pCa/Force relationship

To determine the relation between Ca^{2+} concentration and tension a pCa series is generated by cyclically injecting, at fixed intervals, 88 μl pCa 4.75 solution into the right well of the chamber (R in Fig. 1) while vigorously mixing the solution in R. The same volume of solution that is injected flows across the platform into the left well (L in Fig. 1) where it is removed. With each aliquot of pCa 4.75 injected the pCa of the solution in the right chamber (and on the platform) decreases by an amount that is calculated by serial dilution equations (Brandt et al., 1980). In some experiments the pCa on the platform was monitored with the Ca^{2+}-sensitive electrode. With 30–50 pCa 4.75 injections a complete series of solutions from pCa 8 to near 4.75 is generated.

Experimental protocol

After the cell is skinned it is washed with several ml of pCa 8 relaxing solution and the sarcomere length is set to 2.1–2.2 μm, a length at which the myocyte is not visibly slack and generates no significant resting force. The computer protocol is then started directing injections of pCa 4.75 and data collection. There are 30 or more cycles of pCa 4.75 injections lasting ~40 s each; for the final cycle 10 aliquots of pCa 4.75 are delivered to ensure saturation with Ca^{2+}.

To obtain the true baseline at each steady tension the computer commands the length driver to rapidly (1 ms) decrease the length of the myocyte by a large amount (usually 15–25% l_0) 15 s after each injection then, 4.5 s later, it commands the driver to restretch the myocyte to its previous length. Immediately after the length decrease, while the myocyte is slack, the baseline tension is recorded by the computer. The length changes and the tension are also recorded digitally at 1 kHz by the digital oscilloscope.

The tension data recorded digitally by the microcomputer are compared to those collected by the Nicolet and the chart recorder, the pCa's after each addition are calculated, then the pCa/tension data are fit to the Hill equation.

RESULTS

To find the most gentle method for skinning frog myocytes we tested a number of conditions. The EGTA buffered low Ca^{2+} technique used for mammalian muscle (Wood et al., 1975) failed and we had to add a detergent. The lowest concentration of Triton X-100 that always worked within 5 s was 0.05% in pCa 8 relaxing solution. After the myocytes were mounted between suction micropipettes in relaxing solution a short jet of Triton solution was sprayed on them through a third micropipette. Initially, to confirm that they were skinned, jets of various pCa's from 4.75 to 6 were sprayed on them from other micropipettes (Fig. 3). From this crude series we estimate that 50% maximum force lies between pCa 6 and 5.5.

pCa/Force relationship

After establishing the skinning conditions skinned myocytes were exposed to a pCa series and their force responses recorded. Fig. 4 *A* is the chart record from such an experiment. The top trace is force with large length releases and restretches projecting downward. The upward projections are artifacts from the injection of pCa 4.75. The chart recorder was filtered to remove noise so that the length release to baseline force does not always record completely. It is recorded correctly by the computer and by the Nicolet (see Fig. 4 *B*). The middle trace in Fig. 4 *A* is from the Ca^{2+} electrode. A comparison of the voltage of the Ca^{2+} electrode to calibration voltages (Fig. 4 *C*) shows that the Ca^{2+} concentration according to the electrode is close ($\leq$0.1 p unit) to that calculated with the serial dilution equations. The same result as shown in Fig. 4 *C* is found in all the experiments ($n = 7$) where the Ca^{2+} electrode was used. This confirms that the serial dilution technique works well in the present configuration of the chamber and that there are no major glitches in the smooth delivery of Ca^{2+} to the myofibrils.

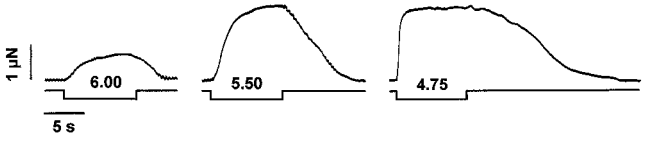

FIGURE 3 Test skinning with three different pCa. Pen records of force (*top trace*) during jetting of Ca^{2+} activating solutions. Skinned myocytes are tested by jetting various pCa on them from a third pipette connected to a pressure reservoir through an electrovalve and positioned close to the myocyte. Bottom traces are the switch signals to the electronic valve controlling the delivery of activating solution. These tests were done only during the development of the skinning technique. Note that the pCa for 50% force lies between 6 and 5.5.

Examples of individual pCa/force relationships obtained from frog atrial and ventricular myocytes are given in Fig. 5. The two smooth curves are drawn from the best fit parameters of the data to the Hill equation. The mean parameters for the pCa/force relationships of all experiments for atrial and ventricular myocytes are given in Table 1. The mean pK of atrial and ventricular myocytes respectively are 5.86 and 5.87, and the n_H's are 4.3 and 5.1. The larger ventricular n_H is not significantly different from that of the atrial myocytes. These slope values are close to those commonly reported for fast skeletal muscle fibers (e.g., Brandt et al., 1980).

In absolute terms the maximal force developed by ventricular myocytes is greater than that generated by atrial cells. The difference, however, can be completely accounted for by the larger cross-sectional area of ventricular myocytes (see Methods). In fact, the normalized maximal force of the two types of preparations is the same (see Table 1).

Force transients following release and restretch

To establish the zero force baseline at each pCa generated by the serial dilution method, a large (15–25% l_0) release, always sufficient to slacken the myocyte, is applied. Typically 4.5 s after the release the myocyte is rapidly restretched to its original length (see Figs. 4 *B* and 6 *A*). One consequence of such long release time is that the myocytes may take up the slack and start redeveloping force before restretch. The extent of force redevelopment at the short length depends on the release extent in any given experiment and on pCa. In those experiments where the largest releases are used (e.g., see Fig. 6 *A*), force redevelopment is usually negligible over a wide range of pCa's and starts becoming evident only at saturating pCa's. In those experiments (e.g., see Fig. 4 *B*) where at the shorter length clear force redevelopment is observed over a range of pCa's, it is evident that threshold and maximum force generation occur at much higher Ca^{2+} than at the prerelease length, consistent with a strong "length dependent activation" (for a review see Allen and Kentish, 1985), and that the slack time depends on pCa, becoming shorter at high Ca^{2+}, in accord with a previous report (Hofmann and Moss, 1992).

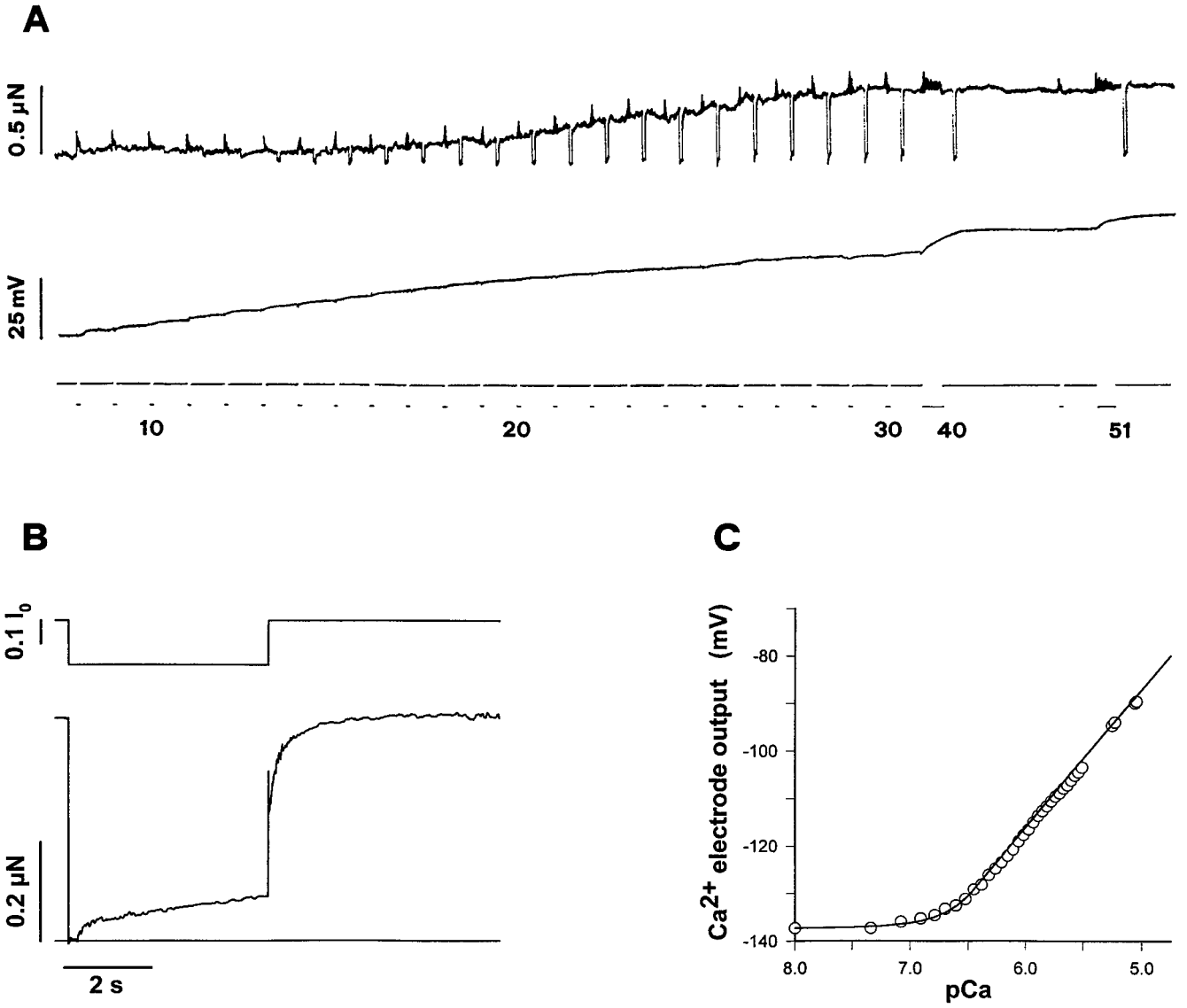

FIGURE 4 Representative experimental records from an atrial myocyte. (*A*) Pen record of force against time (*top trace*), Ca^{2+} sensitive electrode output (*middle trace*), and signals indicating injection of pCa 4.75 into the right well of the experimental chamber (*bottom trace*). The number of pCa 4.75 solution aliquots (88 μl) injected into the chamber is indicated on the lower trace. Upward spikes in the upper trace are artifacts from the Ca^{2+} injection. The downward deflections are force responses to length release and restretch. The gradual increase in force with decrease in pCa is readily apparent. (*B*) Expanded time base of a length release and restretch (*top trace*) for injection 28 of (*A*). The force (*bottom trace*) falls rapidly during the 1 ms release and remains at zero for ~100 ms, then it starts to slowly recover at the shorter length. Upon restretch the force briefly overshoots (phase 1), falls (phase 2), and then redevelops exponentially (phase 3) to the initial level. (*C*) Plot of the calcium electrode output from (*A*) in millivolts on the ordinate (○) against the pCa calculated according to the serial dilution equations (Brandt et al., 1980). The continuous line is the electrode calibration.

With restretch above threshold Ca^{2+} there is a multiphasic force transient (Figs. 4 *B* and 6 *A*) that is independent of the presence and extent of force redevelopment at the shorter length. The sequence of events is always 1) an initial force rise simultaneous with the stretch, 2) a rapid decay, and 3) a slower redevelopment of force to the level before the release. In all experiments the amplitude of phase 1 and the time course of phase 3 changes with the pCa. There is a strong acceleration of the kinetics of phase 3 force redevelopment with increasing Ca^{2+} and increasing steady force (see Fig. 6, *B* and *C*). Fig. 6 *D* shows the correlation between final force levels and the apparent rates of the force redevelopment in phase 3 for 7 atrial and 13 ventricular preparations. The effect of pCa on apparent rate of force generation in phase 3 is large; in ventricular myocytes increasing force from 20 to 100% maximum is accompanied by a 10-fold increase in the apparent rate and in atrial myocytes it is up to 20-fold. The maximal values for the apparent rate of force redevelopment of phase 3 are faster for the atrial myocytes (4.7 s^{-1}) than for the ventricular myocytes (2.7 s^{-1}, Table 2). In Fig. 7 the relationships between pCa and the normalized change in the apparent rate of phase 3 for atrial and ventricular myocytes are compared with the corresponding force-pCa relationships; the appar-

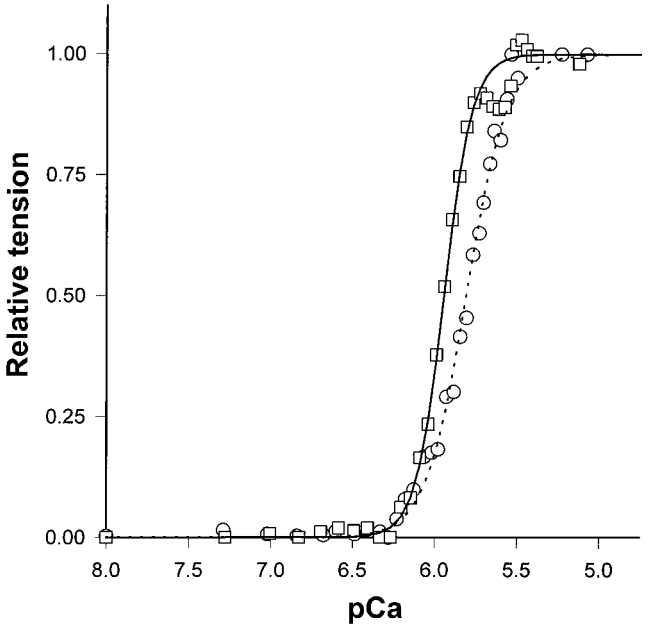

FIGURE 5 pCa/force relationship. pCa/force relationships for individual atrial (○) and ventricular (□) myocytes. The continuous lines are drawn according to the parameters developed by fitting the data to the Hill equation with Origin (Microcal Software Inc. ver. 3.5). pK_{Ca} = 5.81 and 5.95; n_H 3.6 and 5.17 for the atrial (A230694; same experiment as in Fig. 4) and ventricular (V3200695) myocytes, respectively.

ent rate of force redevelopment continues to increase with decrease in pCa even after force saturates (see also tracing at pCa 5.5 in Fig. 6, *A* and *B*).

The mechanism underlying the delayed force response in phase 3 is complex. Besides reattachment of force generating cross-bridges broken by the restretch, length dependent activation or stretch activation may contribute. It is likely, however, that it mainly reflects the kinetics of force generation and can be studied by analyzing the time course of force either after a rapid increase in the activation level (Araujo and Walker, 1994) or following rapid cross-bridge detachment under steady activation (Brenner, 1988). To verify that the kinetics of phase 3 reflects that of force generation, in a few experiments (four atrial and four ventricular myocytes) we reduced the length release to ~10% l_0 and the time interval between release and restretch to between 20 and 40 ms (Fig. 8). This is sufficient to drop force to zero and slacken the myocytes. Before the unloaded myocytes can take up the slack and begin to redevelop force, they are rapidly stretched back to their original length. The force transients produced upon restretch by this procedure are essentially the same as those described above for larger and longer length releases. Analysis of the time course of the delayed force redevelopment in phase 3 shows 1) that the maximum apparent rates of force generation are higher in atrial than in ventricular myocytes (see Table 2) and 2) that the dependence of the rates of force redevelopment on Ca^{2+} is large (see Fig. 8). At pCa 5.85, which is about midway in the pCa/force relationship, the rate constants of force redevelopment following the short release-restretch protocol average 0.96 ± 0.18 s^{-1} and 0.74 ± 0.20 s^{-1} (mean ± SE.; n = 4) in atrial and ventricular myocytes, respectively. These are definitely lower than those measured at maximum activation using the same experimental protocol (see Table 2) and are in reasonable agreement with the data shown at half maximal tension in Fig. 6 *D*.

DISCUSSION

We have made frog atrial and ventricular myofibril preparations by chemically skinning single isolated myocytes held by their tips in suction micropipettes. After skinning one to several myofibrils are left attached at both ends to the pipettes. Using the serial dilution technique (Brandt et al., 1980) we perfuse these with a series of pCa solutions and determine the effects of Ca^{2+} on steady force and on some kinetic properties. The main findings of this study are 1) the high slope of the pCa/force relationship, suggesting a strong cooperativity of Ca^{2+} activation in frog cardiac myofibrils; and 2) the large effects of Ca^{2+} on the rate constant of force redevelopment, suggesting a rather strong kinetic mechanism of force regulation by Ca^{2+}.

We find ~120 kN/m^2 for the maximum tension that can be developed by frog cardiac myocytes, a value that is higher than those commonly reported for mammalian cardiac muscle (e.g., Hibberd and Jewell, 1982; Kentish et al., 1986; Strang et al., 1994) but consistent with those found in skinned *Xenopus* atrial cells (Hofmann and Moss, 1992). The mean active tension found in the present study is somewhat lower than that (149 kN/m^2) recently reported by some of us for the same preparation but using a different experimental protocol (Colomo et al., 1997b). The absolute values of force found here for maximally activated skinned ventricular myocytes are greater than those found for atrial cells because the former contain more myofibrils. In fact, the normalized forces of the two myofibril types are the same, in accord with the supposition that the average force developed by individual myofibrils is the same for both preparations.

TABLE 1 Mean pCa/force parameters

	P_0 (kN m^{-2})	Threshold pCa	Saturating pCa	pK	n_H	n
Atrial myocytes	119.5 ± 11.0	6.19 ± 0.02	5.58 ± 0.03	5.86 ± 0.03	4.30 ± 0.34	7
Ventricular myocytes	123.7 ± 9.7	6.18 ± 0.03	5.56 ± 0.02	5.87 ± 0.02	5.12 ± 0.35	18

Values are means ± SE; n is the number of experiments. Individual experiments were fit using Microcal Origin then the resulting parameters were averaged to get the mean pK_{Ca} and n_H. Also given are the mean maximum forces (P_0) normalized to cross-sectional area, the thresholds (≤5% P_0), and saturating pCa levels (≥95% P_0).

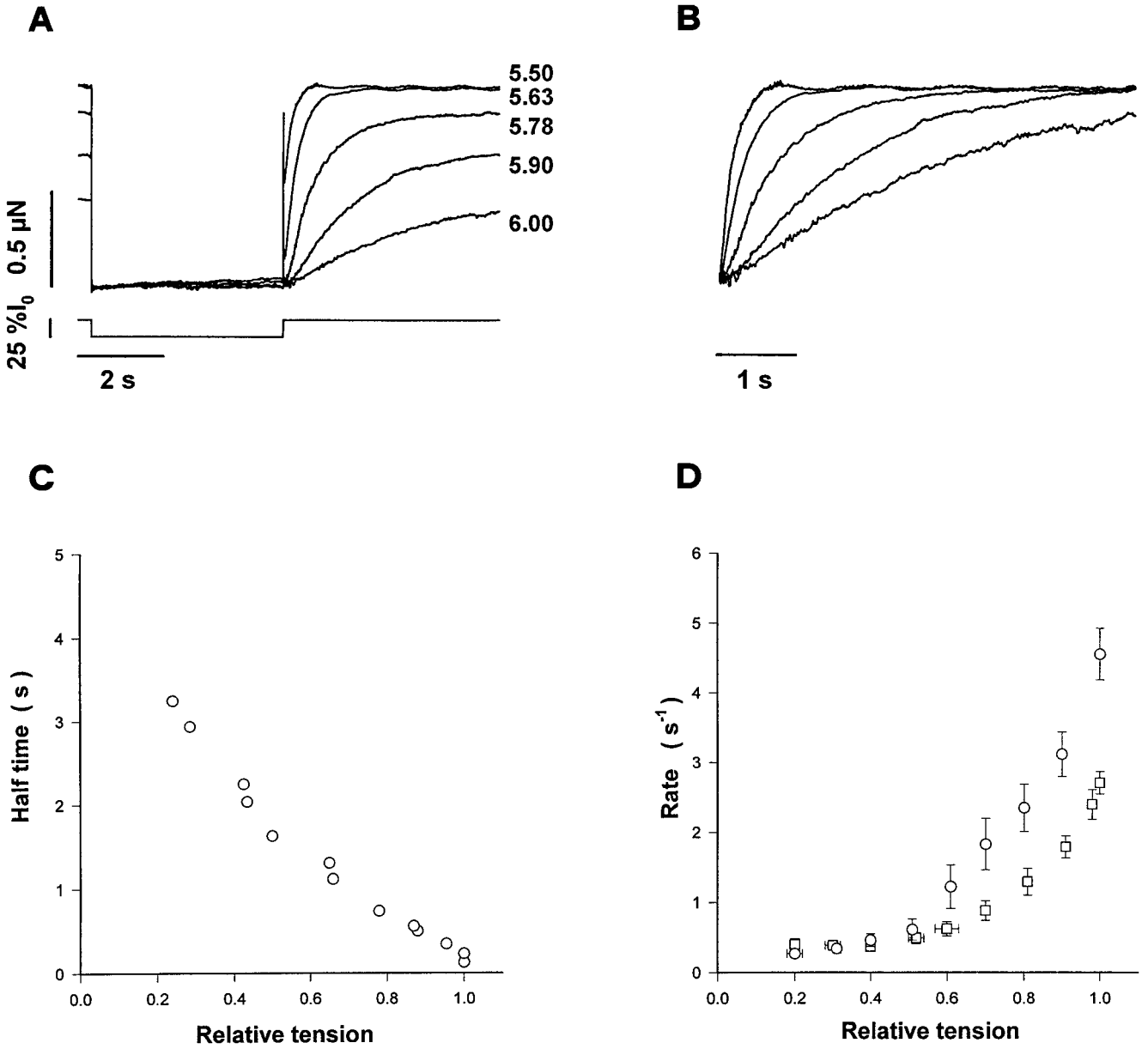

FIGURE 6 Kinetics of force redevelopment on restretch. (*A*) Experimental records of the force responses following length release and restretch for a single atrial myocyte. pCa levels indicated. Lower trace is myocyte length. (*B*) Same force traces as in (*A*) with each normalized to its maximum to show more clearly changes in the half time of force redevelopment with pCa. (*C*) Same experiment as (*A*) showing the relation between relative force and half-time of force redevelopment upon restretch. (*D*) The mean rate constants for 7 atrial and 13 ventricular myocytes plotted against relative force. Rate = 1/(half-time × 1.44). The error bars are the standard error of the means. For analysis the data are grouped into 0.10 relative force blocks.

pCa/Force relationship

The first study of chemically skinned cardiac muscle was done by Winegrad (1971) who exposed strips of frog ventricle to an EDTA solution, which opened the membrane barrier to small ions. The midpoint of his pCa/force relationship (Fig. 3) is ~pCa 5.8 and the slope or n_H is ~1. Fabiato and Fabiato (1978) mechanically isolated frog ventricular myofibril bundles and perfused them with a series of pCa solutions; the midpoint of their pCa/force curve is ~6.0 and the n_H is ~1.8. Rat right ventricular trabeculae, skinned by exposure to Brij-58 (1%) for 30 min, give a mean pK_{Ca}

TABLE 2 Maximum rates of force redevelopment following release-restretch protocols

	Atrial Myocytes	Ventricular Myocytes
Large release protocol	$4.56 \pm 0.37\ s^{-1}$ ($n = 7$)	$2.73 \pm 0.14\ s^{-1}$ ($n = 18$)
Short release protocol	$5.27 \pm 0.80\ s^{-1}$ ($n = 4$)	$2.82 \pm 0.32\ s^{-1}$ ($n = 4$)

Values are means ± SE; the numbers of experiments (n) are given in parenthesis. Rate = 1/(half time × 1.44). The large release protocol length change ranged between 15 and 25% l_0 and the duration of the release was 4.5 s. The short release protocol length change was 10% l_0 and the duration ranged between 20 and 40 ms.

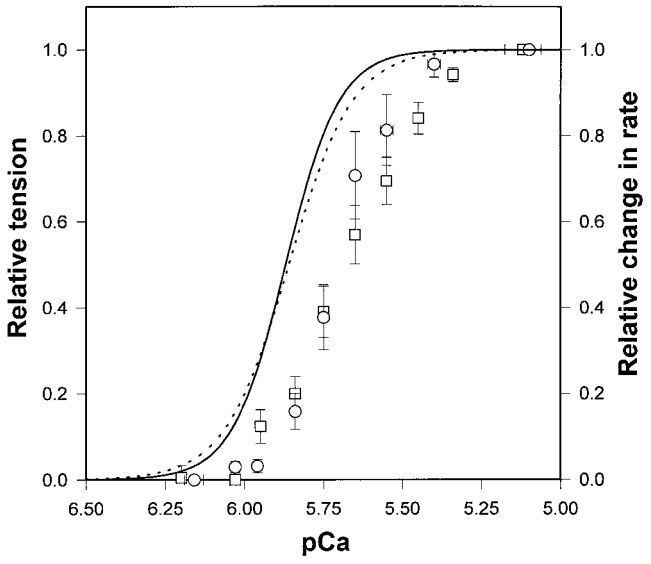

FIGURE 7 Effects of pCa on force and on rate of force redevelopment. The mean normalized pCa/force relations for ventricular (*solid line*) and for atrial (*dotted line*) myocytes are compared to the mean normalized relations between pCa and rate constant of force redevelopment after restretch for atrial (○) and ventricular (□) myocytes. The lines on the rate constant data points are the SEMs. The minimum rates at the lowest activation levels were subtracted to better show the Ca^{2+}-dependent changes. Whether or not this subtraction was made the rates are displaced to the right of the force curves. This is because the rates continue to rise after the force saturates.

of 5.5 and an n_H of 2.5 (Hibberd and Jewell, 1982). *Xenopus* atrial myocytes, skinned by exposure to saponin for 30 min and knotted by their ends to microbeads (Hofmann and Moss, 1992), give (in the presence of 10 mM caffeine) a mean pK_{Ca} of 6.16 and an n_H of 2.20. Rat cardiac myocytes, glued to glass microtools and made permeable by α-hemolysin (Sweitzer and Moss, 1994), yield a midpoint of pCa 5.6 and n_H of 2. Rabbit ventricular single myofibrils (Linke et al., 1994) yield a midpoint of pCa 5.5 and an n_H of 1.92. The pK_{Ca} reported here, 5.86, is centered in the range of all these previous studies; we find, however, an n_H of 4.3 for atrial cells and 5.1 for ventricular cells. This is much larger than those reviewed above and significantly greater than 2, so it can be argued that the cooperativity of Ca^{2+} activation in frog heart myofibrils involves at least several neighboring regulatory units (one Tropomyosin-Tn and seven actins).

One main difference in our study from those of others is that we have a robust method of attaching the myofibrils to the apparatus that does not damage them by crushing or gluing; the myofibrils are drawn into a suction pipette while they are still in an intact myocyte. Perhaps the attachment of the myofibrils to the myocyte membrane is transferred to the glass holding pipette in a way that preserves more normal structure. Kentish et al. (1986) reported an n_H of 4.5 for skinned rat ventricular trabeculae contracting at a constant sarcomere length; they suggested that, because of the large effects of sarcomere length changes on the Ca^{2+} sensitivity of cardiac muscle, large end compliance can artificially decrease the slope of the pCa/force relation. Although we do not measure sarcomere length during contraction, in our experimental conditions the series compliance of frog heart myocytes is small (see Colomo et al., 1994); at maximal activation, the minimum shortening required to drop tension to zero is usually much less than 5% l_0 (data not shown but see Fig. 8). The small end compliance should better reveal high n_H values intrinsic to the myofibrils than experiments with larger end compliances.

Nonetheless, we cannot measure or control sarcomere length simultaneous with force measurements, so we cannot completely exclude the possibility that our results are affected by some mechanical artifacts. In particular, it could be argued that end compliance in our preparations is greatest at low $[Ca^{2+}]$ and least at high $[Ca^{2+}]$. This Ca^{2+} dependent end compliance can produce larger mechanical effects at low than at high activation and can artificially increase the slope of the pCa/force curve (as well as the Ca^{2+} effect on force redevelopment; see below). This possibility is unlikely because in our experiments the minimum shortening required to drop myocyte tension to zero decreases with decreasing $[Ca^{2+}]$ (data not shown). Internal shortening, therefore, cannot be any greater at low than at high levels of activation, which makes the artifact suggested above unrealistic. Furthermore, the symmetry of the pCa/force curves observed in this study seems to preclude any large artifacts due to end effects. At variance with many previous results (e.g., Brandt et al., 1980; Sweitzer and Moss, 1990), we find the pCa/force relation is best fit by a single sigmoid curve (Fig. 5) rather than by two of different slope joined at the midpoint. Recent results from Moss's laboratory (Sweitzer and Moss, 1994) are also best fit by a single symmetrical curve. Related experiments (Brandt and Linari, unpublished results) show that when sarcomere length is kept constant the pCa/force relationship is symmetrical about the midpoint, and suggest that disorganization of the myofibrils at their attachment to the apparatus accounts for at least some of the earlier two-phase pCa/force curves.

A number of other factors may also help determine the high slopes of the pCa/force curves found in this study. At variance with most of the other reports, we do not average together data points from different curves but calculate the Hill parameters for each experiment separately and average the parameters. This will increase n_H over that found by averaging points before fitting (Brandt et al., 1980). It is also possible that the slope of the pCa/force relationships found here are high in part because our solutions contain 87 mM free Mg^{2+} while those of others are often 1 mM or higher. Low free Mg^{2+} has been reported to increase the steepness of the pCa/force curve (Best et al., 1977), although the opposite result has also been found (Zot and Potter, 1987). The procedure we use to change $[Ca^{2+}]$ differs from those used by others. In principle, the serial dilution method is good in the steady state but, during transitions to higher $[Ca^{2+}]$, it may be susceptible to artifacts due to slow mixing. The results of the experiments

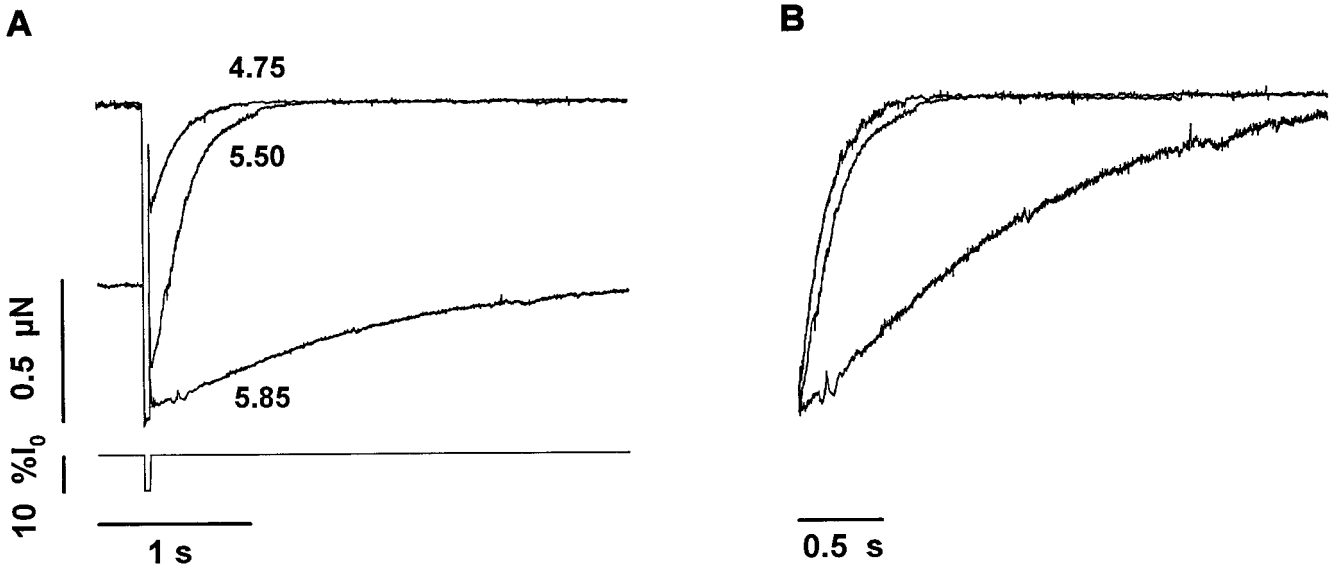

FIGURE 8 Short release-restretch protocol. (*A*) Experimental records of force (*top traces*) following a 10% l_0 release and restretch (*bottom trace*). Three different pCa levels are shown. Although the interval between the length changes is shorter (35 ms) than the others reported here (4.5 s), the results are similar. This demonstrates that longer intervals between length release and restretch do not materially affect the time course of force recovery. (*B*) Same traces as in (*A*) with each force normalized to its maximum.

with the Ca^{2+} electrode (e.g., Fig. 4, *A* and *C*) demonstrate that Ca^{2+} is delivered smoothly to the myocytes and seem to preclude the possibility that the Ca^{2+} sensitivity of the preparations is affected by transient exposure to high [Ca^{2+}] before mixing is complete. Moreover, pCa/force data points obtained on the same preparations by individual hits of premixed solutions (e.g., see Fig. 3) are consistent with those found with the serial dilution method, although the former data points are too few for a meaningful estimate of the n_H. Finally, our procedure also differs from that of most other studies in that we progress through the pCa series without relaxing the myofibrils or mechanically shocking them by a solution change between each pCa increment, although we release the preparation length to determine the baseline after each pCa step.

Rate of force redevelopment

The maximum values found here for the apparent rate of force generation in both types of frog cardiac myocytes (Table 2) are similar to those of slow muscles (Metzger and Moss, 1990), while the difference between atrial and ventricular myocytes suggests that the intrinsic speed of the contractile proteins is higher in the frog atria than in the ventricle. This is in agreement with results from mammalian hearts where differences in both myosin heavy and light chains are thought to contribute to the higher speed of contraction of atrial versus ventricular myocardium (Bottinelli et al., 1995). It is not known which myosin is expressed in frog myocytes. However, immunohistochemical staining and immunoblotting analysis with different anti-myosin heavy chain monoclonal antibodies indicate that the heavy chain isoforms expressed in the frog atria differ substantially from those in the ventricle (D. Mornet, personal communication).

The present study of frog cardiac myofibrils supports regulation by Ca^{2+} of the kinetics of force redevelopment. In most of our experiments length release was large and its duration long compared to that usually employed to study the kinetics of force generation. This protocol was adopted to yield a long-lasting zero force baseline at each pCa. Although this protocol is not ideal for studies of force generation kinetics, the strong dependence of the rate of force redevelopment on Ca^{2+} has been confirmed by an additional, more orthodox, release-restretch protocol (Fig. 8). In accord with the present result, we find (Colomo et al., unpublished data) that the rate constant of force rise following rapid activation of frog atrial myofibrils by solution changes complete in $<$10 ms (Colomo et al., 1997a) undergoes at least a 10-fold increase with decrease in pCa from 5.75 to 4.75. It is unlikely that the extent of the Ca^{2+} effect we observe can be explained by compliance end effects (see above).

In mammalian fast skeletal muscle the rate of isometric force redevelopment following a release-restretch protocol is also strongly influenced by the level of Ca^{2+} (Brenner, 1988; Metzger and Moss, 1990; Millar and Homsher, 1990; Sweeney and Stull, 1990; Chase et al., 1994). In contrast, the apparent rate of force generation in mammalian cardiac muscle is relatively insensitive to Ca^{2+}, ranging from a threefold (Wolff et al., 1995) to no change (Hancock et al., 1993, 1996). In general, the rate of force development is less Ca^{2+} sensitive in slow than fast skeletal muscle fibers (Metzger and Moss, 1990). In the present study the rate of force redevelopment of frog cardiac myofibrils varies with Ca^{2+} by $>$10-fold, a range similar to that reported for

mammalian fast skeletal muscle. We conclude that the mechanism behind Ca^{2+} regulation of force redevelopment is not a function of the intrinsic speed of the contractile apparatus. The conclusion is strengthened by some preliminary results obtained on single myofibrils from frog fast skeletal muscle (Colomo et al., 1997a). In contrast to the steep dependency of force redevelopment on Ca^{2+} found here in frog cardiac myofibrils, frog tibialis anterior myofibrils at 15°C exhibit little dependency of the kinetics of force generation on Ca^{2+}, like that reported by others for mammalian cardiac muscle. Why such diverse muscle types should be so paired is perplexing.

Mechanism of Ca^{2+} sensitivity

Initially there was debate about the effect of Ca^{2+} on the kinetics of the cross-bridge cycle (Podolsky and Teicholtz, 1970; Julian, 1971) but much evidence has since accumulated that Ca^{2+} directly or indirectly affects the rates inferred to the cycle (Brenner, 1988; Metzger and Moss, 1990). Certainly our results on small groups of frog cardiac myofibrils show a strong dependence of force redevelopment on Ca^{2+}. What is unresolved is by what mechanism Ca^{2+} produces an apparent effect on cycle kinetics, if its effect is direct, as first proposed by Julian (1969), or indirect, as proposed by some more recent schemes (e.g., Campbell, 1997). Any proposed mechanism should also explain why the extent of the Ca^{2+} effect varies among different muscle types.

While there appears to be little Ca^{2+} dependence of any cross-bridge kinetic transition that has been studied so far, Campbell's scheme (1997) provides a mechanism by which the extent of the Ca^{2+} effect on force redevelopment varies with the degree of cooperativity of the pCa/force relationship. In fact, according to Campbell's scheme, both cooperativity of the pCa/force relationship and the indirect effect of Ca^{2+} on cycle kinetics result from feedback of force generating cross-bridges on Ca^{2+} activation of thin filaments. This scheme seems, therefore, able to explain the present findings on frog cardiac myofibrils (steep slope of the pCa/force relationship, large Ca^{2+} effect on force redevelopment). However, according to Campbell's generalized mechanism, the first part of the pCa/force relationship will have a steeper slope than the last part at low pCa. We find no change in slope through the pCa/force relationship (Fig. 5 and Table 1). Our individual data sets fit the Hill equation well in both the lower and upper segments of the curve. Because the n_H is as high as reported for skeletal muscle, it is unlikely that slope change is masked by a low initial slope. In this respect, our results are not in accord with the indirect effect proposed by Campbell (1997). Evidence that cooperativity in the pCa/force relationship may not be coupled to the effect of Ca^{2+} on the rate of force generation has also been reported for skeletal muscle; TnC extraction greatly reduces the steepness of the pCa/force relationship but does not reduce the effect of Ca^{2+} on the rate of force redevelopment (Metzger and Moss, 1991).

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