Approximating the Isometric Force-Calcium Relation of Intact Frog Muscle Using Skinned Fibers

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ABSTRACT In previous papers we used estimates of the composition of frog muscle and calculations involving the likely fixed charge density in myofibrils to propose bathing solutions for skinned fibers, which best mimic the normal intracellular milieu of intact muscle fibers. We tested predictions of this calculation using measurements of the potential across the boundary of skinned frog muscle fibers bathed in this solution. The average potential was −3.1 mV, close to that predicted from a simple Donnan equilibrium. The contribution of ATP hydrolysis to a diffusion potential was probably small because addition of 1 mM vanadate to the solution decreased the fiber actomyosin ATPase rate (measured by high-performance liquid chromatography) by at least 73% but had little effect on the measured potential. Using these solutions, we obtained force-pCa curves from mechanically skinned fibers at three different temperatures, allowing the solution pH to change with temperature in the same fashion as the intracellular pH of intact fibers varies with temperature. The bath concentration of Ca^{2+} required for half-maximal activation of isometric force was 1.45 μM (22°C, pH 7.18), 2.58 μM (16°C, pH 7.25), and 3.36 μM (5°C, pH 7.59). The $[Ca^{2+}]$ at the threshold of activation at 16°C was ~1 μM, in good agreement with estimates of threshold $[Ca^{2+}]$ in intact frog muscle fibers.

INTRODUCTION

Previously we (Godt and Maughan, 1988) compiled a list of the likely intracellular constituents of intact frog muscle and found that electroneutrality dictated a substantial concentration of negative charge that could not be accounted for by known diffusible anions. We proposed that this negative charge was likely to be supplied by the fixed myofibrillar proteins. At physiological pH these proteins bear a net negative charge, whose magnitude, estimated from amino acid analysis, is consistent with that required for the overall electroneutrality of the intracellular medium.

In a later communication (Maughan and Godt, 1989), we developed a mathematical description of the equilibrium (Donnan) distribution of diffusible ions across the boundary of a myofibril containing such fixed charges. Given our earlier compilation of the principal ionic constituents (Godt and Maughan, 1988; see table 1 in Maughan and Godt, 1989), we calculated the magnitude of the net negative fixed charge and of the Donnan potential that would be found across the myofibrillar boundary. Moreover, this model allowed us to design a bathing solution for skinned muscle fibers in which the intramyofibrillar ion concentrations closely approximate those found in resting, intact frog muscle cells. Our aim was to provide a solution in which the myofibrils are not disturbed by skinning.

In the present paper we measure the potential across the boundary of skinned frog muscle fibers bathed in such a bathing solution and compare this experimental value with that predicted in the Donnan model. We find that the actual value is similar to that predicted, thus supporting the previous analysis. Under these conditions, the contribution of any diffusion potential arising from the differences in the mobilities of charged substrates and products of ATP hydrolysis within the skinned fiber (Godt and Baumgarten, 1984) is small, since inhibition of myofibrillar ATPase with vanadate did not appreciably affect the measured potential. We then measured the relationship between isometric force and bath $[Ca^{2+}]$ in a solution mimicking the intracellular medium, thus providing an accurate estimation of calcium dependence of contractile activation in intact frog muscle.

MATERIALS AND METHODS

Preparation and experimental solutions

Frogs (*Rana temporaria*) were obtained from Charles D. Sullivan Co. (Nashville, TN) and were kept for at least a week in a refrigerator before use. All experiments were conducted on segments of single fibers dissected from the semitendinosus muscle. Fiber segments were chemically skinned using 0.5% v/v purified Triton X-100 detergent (Boehringer Mannheim, Indianapolis, IN) in a relaxing solution (pCa > 8.5; see below). After skinning, striation spacing, measured from the diffraction pattern produced by a He-Ne laser, was set at 2.0 μm.

Based on the analysis of Maughan and Godt (1989), the following physiological intracellular solution approximates the primary conditions of the cytosol to which the myofilaments are exposed in the relaxed, intact muscle: 54 mM dipotassium phosphocreatine (K_2PCr), 0.25 mg/ml (~87.5 units/ml) creatine kinase, 6.8 mM K_2ATP, 10.8 mM $Mg(OH)_2$, 18.6 mM carnosine, 1 mM parvalbumin, 1.5 mM phosphoric acid, ~ 8.8 mM HCl to bring pH to 7.18 at room temperature, and 4 g/100 ml Dextran T-500. The calculated free $[Mg^{2+}]$ is 1.54 mM, $[Mg_2ATP]$ is 5.91 mM, and ionic

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strength is 194 mM. Glyolytic intermediates, amino acids, and diffusible proteins (primarily parvalbumin and glycolytic enzymes) are also normal constituents of the cytosol but were omitted for simplicity because their total electrostatic contribution to the Donnan potential is small compared to the total from the constituents listed above (Godt and Maughan, 1988). In the present study, fibers were bathed in a physiological intracellular solution (abbreviated PIS) of the same composition as that of the solution above, except that 54 mM Na_2PCr was substituted for the more expensive dipotassium salt. This switch of major cation has no known functional consequences at physiological ionic strength (for $\mu > 0.15$ M, the magnitude of Ca^{2+}-activated tension is independent of whether Na or K is used as the primary cation) (Gordon et al., 1973). Furthermore, 2 mM EGTA was substituted for 1 mM parvalbumin as the Ca^{2+} buffer (both have similar buffering capacity). The present solution also differs slightly from that given in Maughan and Godt (1989) in that the total magnesium concentration was decreased from 12 to 10.8 mM to reflect the differences between Mg binding to EGTA and parvalbumin, and the high-molecular-weight dextran was added to maintain in vivo myofilament lattice spacing (Godt and Maughan, 1981; Millman, 1981). Solutions with various free calcium concentrations were obtained by addition of $CaCl_2$. Binding constants used in the computations of solution composition are given in Godt and Maughan (1988) and Godt and Lindley (1982). These constants imply the following values for the apparent dissociation constant of EGTA for calcium at the indicated temperatures and pHs: 1.86×10^{-7} (22°C, pH 7.18); 1.40×10^{-7} (16°C, pH 7.25); 3.31×10^{-8} (5°C, pH 7.59).

Solutions were cooled to 5–6°C, at which point the pH was 7.59. The pH buffer used (carnosine) is a close approximation to the native soluble pH buffers (Godt and Maughan, 1988). When temperature is changed in vivo, a new intracellular pH is immediately achieved, most likely because of the temperature dependence of the pKs of the intracellular buffers (Godt and Maughan, 1988; Marjanovic et al., 1994). Long-term maintenance of intracellular pH near neutrality suggests that the set-point of the Na^+-H^+ exchanger must also vary with temperature (Marjanovic et al., 1994). Thus the change of pH of PIS with temperature probably reflects changes in vivo of intracellular pH in cooled frog muscle (Malan et al., 1976; Dawson and Elliott, 1985; Marjanovic et al., 1994).

Potential measurements

Small bundles of fibers were cut from the muscle and placed in PIS (see above) in a dish whose bottom was thinly coated with Sylgard (Dow Corning Corp., Midland, MI). The temperature of the dish was maintained at 5–6°C with a Peltier device (Cambion, Cambridge, MA). Single fibers were separated from the bundle, skinned mechanically using fine needles, and pinned out straight with small insect pins. Purified Triton X-100 detergent was added to the bath to a final concentration of 0.5% v/v. After 30 min the solution was replaced with one free of Triton. Triton was included to eliminate mitochondria and sarcoplasmic reticulum (Aldoroty and April, 1984). The potential difference across the skinned fiber boundary was recorded with conventional 3 M KCl-filled glass microelectrodes (10–20 Mohm) connected to a high-input resistance, unity-gain amplifier (W.P. Instruments, New Haven, CT), using a standard calomel reference electrode. Signals were monitored on a chart recorder and a digital voltmeter with 0.1 mV resolution. Electrode resistance was monitored periodically to ensure that the electrodes were stable. The criteria for successful introduction of the electrode tip into the myofibrils (impalement) are given in Godt and Baumgarten (1984). The optics did not permit accurate positioning of the electrode tip within the sarcomere. Given that the thick filaments are 1.6 μm long (Gordon et al., 1966) and that the average sarcomere length of the fibers was 2 μm, 80% of the random impalements were likely to be in the A-band and 20% in the I-band. There was little probability of impaling the very narrow (0.05 μm) Z-line.

Elliott and Bartels (1982) examined the theoretical and practical limitations of measuring Donnan potentials in extended hexagonal gels (such as demembranated muscle). They reasoned, as Collins and Edwards (1971) and Elliot et al. (1978) had earlier, that the potentials measured resulted from the fixed electric charge on the muscle myofilament lattice. Naylor (1982) calculated the magnitude of the interfilament potential that would be predicted using an approach independent of that used by Elliot and Bartels (1982) and concluded that the interfilament space is a regime in which potential gradients produced by the fixed myofilament charge can be treated as simple Donnan averages.

Experiments on agar gels (Collins and Edwards, 1971; Aldoroty and April, 1984), surrogates for skinned striated muscle fibers, provide positive controls for interpreting the potential measurements in the context of Donnan theory. As in the present experiments, Donnan potentials were measured using KCl salt bridges, because no work (and therefore no potential) can be measured from a system that is in equilibrium. Although salt bridges introduce a liquid junction potential (Overbeek, 1956), the measured potentials were not significantly different when (1) the salt concentration in the electrode was varied (1–3 M), (2) the tip diameter (resistance) of the electrode was varied (13–30 Mohm), or (3) when K propionate was substituted for KCl (either in the bathing medium or within the gel) (Aldoroty and April, 1984). Furthermore, the Donnan potential equation (e.g., eq. 13 in Maughan and Godt, 1989) successfully predicted the measured electrochemical potentials as a function of fixed charge density in agar gels (i.e., agar concentration; Aldoroty and April, 1984) and crayfish skinned muscle fibers (i.e., myofilament lattice volume; Aldoroty et al., 1987). Finally, agreement between the predicted and observed relationship between electrochemical potential and ionic strength has been demonstrated for the agar gel system (April and Aldoroty, 1986; Maughan et al., 1991) as well as for crayfish (April and Aldoroty, 1986), barnacle (Stephenson et al., 1981), and frog (Maughan et al., 1990) skinned muscle fibers. Results such as these demonstrate conclusively that artifactual diffusion potentials from KCl salt bridges do not contribute significantly to the measured electrochemical potentials.

Measurement of fiber ATP hydrolysis

ATP hydrolysis of the skinned fiber segments was estimated from the resultant build-up of ADP in a 5- or 10-μl drop of PIS. To ensure that ADP production was tightly linked with ATP hydrolysis, reactions catalyzed by creatine kinase and adenylate kinase had to be prevented. This was accomplished by pretreating each fiber for 20 min with 0.38 mM 2,4-dinitrofluorobenzene (Sigma, St. Louis, MO) to irreversibly inhibit endogenous creatine kinase activity (Infante and Davies, 1965) and by including 10 μM diadenosine pentaphosphate to inhibit adenylate kinase (Feldhaus et al., 1975). Fibers were then incubated in the test drops for 30 min, after which the ADP concentration in each drop was measured using high-performance liquid chromatography (model 6000A solvent delivery system and model 440 absorbance detector, Waters, Milford, MA). Creatine, creatine phosphate, AMP, ADP, and ATP were separated on a reverse-phase column (Biophase ODS, 5 μm, Bioanalytical Systems, West Lafayette, IN) as described by Victor et al. (1987). Two microliters of experimental solution was mixed with 8 μl of running buffer and injected into a 20-μl loop (7125 sample injector, Rheodyne, Cotati, CA). Optical absorbance was measured at 214 nm, and all peaks were detected within 10 min of sample injection (pump speed, 3 ml/min; running buffer, pH 4.0). The ATP/ADP concentration ratio was determined from the peak heights (after making a peak height adjustment for retention time). "No-fiber" control drops were also analyzed and background hydrolysis was subtracted. Net ATP hydrolysis rates were normalized per mole S-1 from measurements of fiber length and width determined using a stereo microscope equipped with a filar micrometer (LaSico, Los Angeles, CA). S-1 concentration was taken to be 0.28 mM (Ferenczi et al., 1978).

Isometric force measurements

The force-pCa relationship of detergent-skinned fiber segments was measured as described by Godt and Nosek (1989). Temperature of the bathing solutions was set at 22°C (pH 7.18) using flowing coolant. We also determined the force-pCa relationship in the same set of solutions cooled to 16°C (at which point pH equaled 7.25) and 5–6°C (pH 7.59).

Statistics

Analysis was performed using commercial statistical software (SYSTAT, Evanston, IL). Statistical significance was decided using $p < 0.05$ as the criterion (Tukey HSD test; Bartlett's test for homogeneity of variance).

RESULTS

Potentials in frog PIS

Fig. 1 shows the distribution of potentials measured from random impalements in relaxed skinned frog muscle fibers bathed in PIS (pCa > 8.5). The measured potentials (hereafter denoted U_m) were distributed about a mean of −3.1 mV (+2.0 mV SD; 365 impalements in 60 fibers from 28 frogs), which is not significantly different from the U_m predicted previously (−2.7 mV) using our Donnan distribution model and literature values for cytoplasmic constituents (Maughan and Godt, 1989).

Vanadate decreases resting actomyosin ATPase but has little effect on U_m

If a diffusion potential arising from ATP hydrolysis by the fiber contributes significantly to the measured potentials, diminution or elimination of ATP hydrolysis should alter the potential. Because our skinned fibers were devoid of mitochondria and sarcoplasmic reticulum due to pretreatment with Triton X-100, any ATPase remaining will be that of actomyosin. Preclusion of actomyosin ATPase by removing ATP from the bathing solution leads to a large increase in the measured potential in skinned muscles from every species tested (see Aldoroty and April, 1984, their table 4), including frog (Godt and Baumgarten, 1984). However, this does not argue unequivocally for the presence of a diffusion potential in ATP-containing media because removing ATP causes large changes in the structure of the thick filament (Clarke et al., 1986; Ashton et al., 1988; Padron and Craig, 1989), which might alter the net charge density (see Bartels and Elliott, 1985, for other possibilities). Thus we tested for diffusion potentials by inhibiting ATP hydrolysis with vanadate.

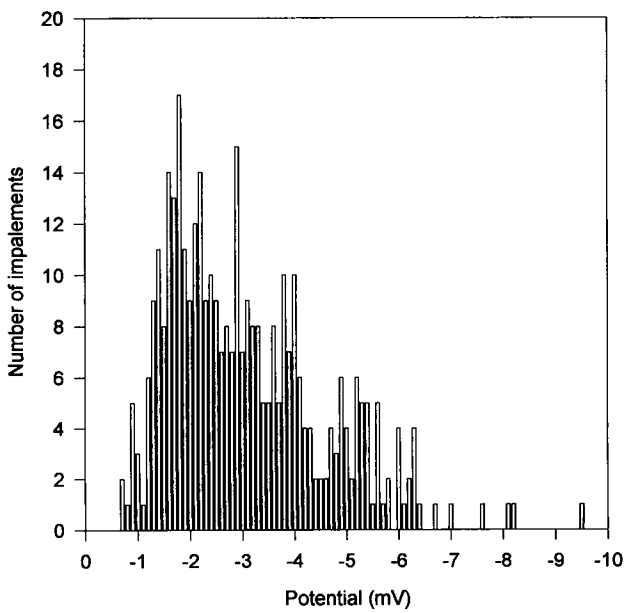

FIGURE 1 Histogram of potentials in skinned frog muscle fibers bathed in physiological intracellular solution at 5–6°C. Bar width, 0.1 mV.

Goodno and Taylor (1982) found that 1 mM orthovanadate inhibits actomyosin ATPase in vitro, because of the formation of a stable myosin·ADP·vanadate complex. Dantzig and Goldman (1985) suggested that, in a skinned fiber, vanadate does not bind to myosin until the fiber is activated by Ca^{2+}, i.e., under conditions where the cross-bridges cycle. Cross-bridges do cycle in relaxed fibers ($Ca^{2+} < 10^{-8}$ M), albeit slowly. We reasoned that, given enough time, vanadate might bind to myosin in relaxed fibers and thereby suppress actomyosin ATPase. To test this possibility, we compared the rate of ATP hydrolysis of frog skinned fibers in PIS, with and without 1 mM vanadate. Table 1 shows that 30 min of incubation in 1 mM vanadate significantly reduces resting fiber ATPase, to about one-quarter of its level measured over an equivalent period before vanadate treatment. This is somewhat greater than the inhibition of rabbit acto·S-1 ATPase activity observed by Goodno and Taylor (1982) at 1 mM vanadate (their figure 2). Suppression of fiber ATPase by vanadate may actually be larger because, in the absence of vanadate, the resting ATPase increased with time (as has also been observed in *Lethocerus cordofanus* skinned muscle fibers in Ca^{2+}-free solutions; Pybus and Tregear, 1975). This time-dependent increase is about twofold over a period of an hour, i.e., over a time interval that includes both the pre-vanadate control and vanadate test measurements. Thus a 30-min exposure to relaxing solution with 1 mM vanadate reduces fiber actomyosin ATPase to between one-quarter and one-eighth its normal resting rate.

With this in mind, we compared U_m before and after a 30-min incubation in PIS containing 1 mM vanadate. The results are shown in Table 1. Compared to the pre-vanadate control, vanadate caused a small decrease in U_m at the borderline of significance ($p = 0.049$). No significant change in U_m was observed when the fibers were returned to control PIS. This is not unexpected because washout of

TABLE 1 Effect of 1 mM vanadate on average resting ATP hydrolysis rates and potential (U_m) in Triton-skinned muscle fibers of the frog

Solution	ATPase (pmol/pmol S1/s)	U_m (mV)
PIS pre-vanadate control	0.011* (0.004, 20)‡	−3.8 (1.4, 66)
PIS + 1 mM vanadate	0.003§ (0.002, 9)	−3.4 (1.3, 68)
PIS post-vanadate control		−3.6 (1.5, 73)

*Initial 30-min control period. ATPase rate rose to 0.017 pmol/pmol S1/s (0.004, 9) in subsequent 30-min control period.
‡Standard deviation, number of observations.
§Significantly different than control ($p < 0.05$).

vanadate from skinned muscle fibers in relaxing solution is quite slow, on the scale of minutes to hours (Dantzig and Goldman, 1985), and most of the measurements of U_m after vanadate were made within ~ 1 hr. Thus, these data suggest that if an ATP-dependent diffusion potential exists under our conditions, it is small (−0.4 mV) and can be neglected in our calculations.[1] The pre-vanadate control data also suggest that our PIS solution adequately mimics normal intracellular solution.

Validation of solution composition

The similarity of U_m to the value predicted on the basis of known properties of the intracellular milieu (Maughan and Godt, 1989) confirms that PIS mimics native cytosolic solution of muscle at rest in at least one important respect. That is, the ionic constituents of PIS, all of which are native to or similar to known constituents of frog muscle cytosol, maintain a fixed (myofilament) charge density similar to that which probably exists in vivo. The similarity of observed and predicted values argues against the existence of large solution differences in ionic strength or pH, both of which affect Donnan potentials by screening or titrating fixed charge (~42 meq/l; Maughan and Godt, 1989). Although the method does not allow us to directly assess differences in type or concentration of specific ion species, especially those important physiologically,[2] the present results are compatible with estimates of free concentrations of Mg^{2+}, K^+, Na^+, $MgATP^{2-}$, etc. tabulated in table 2 of Maughan and Godt (1989).

Force-calcium relationship

Assuming that PIS is an accurate representation of the ionic medium experienced by myofibrils in intact frog muscle, we used these solutions to generate the force-pCa relationships shown in Fig. 2. The calcium concentration for half-maximal activation (Ca_{50}) and the Hill coefficient (N) fit to those data are listed in Table 2. The curves in Fig. 2 are plotted relative to the calcium concentration in the bath. However, Ca^{2+} in the myofibrillar space will be higher because of the net negative charge on the myofilaments. Using the Nernst equation, we calculated previously that, on average, the concentration of divalent cations will be 1.234 times greater in the intramyofibrillar phase than in the extramyofibrillar phase (table 3 in Maughan and Godt, 1989). When this factor is used, the plot of force versus average calcium concentration in the myofibrillar space is shifted rightward by 0.09 log units (log 1.234), as shown by the lower abscissa in Fig. 2.

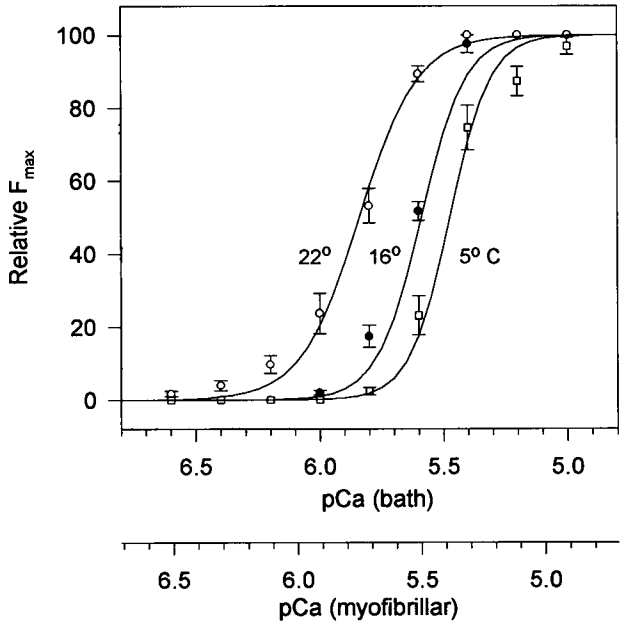

FIGURE 2 Normalized force-pCa relationship of skinned frog muscle fibers bathed in Physiological Intracellular Solution at the temperatures indicated. Data points at each pCa are averages of 6 (22°C), 9 (16°C), and 7 (5°C) fibers. Data from each fiber were fit to the Hill equation ($\%F_{max} = [Ca^{2+}]^N/(Ca_{50}{}^N + [Ca^{2+}]^N)$), where Ca_{50} is the calcium concentration in the bath (upper abscissa) required for 50% activation and N is a measure of the slope. The curves plotted are from Hill equations using the average N and Ca_{50} for each temperature (see Table 2). Lower abscissa shows calculated average $[Ca^{2+}]$ in the intramyofibrillar space. The absolute value of maximum calcium activated force decreased with temperature such that, relative to 22°C, it was 92.3% (±2.4% SD) at 16°C and 86.1% (±2.9% SD) at 5°C. These changes correspond to a Q_{10} of 1.17 (16–22°C) and 1.07 (5–16°C), slightly smaller than the Q_{10} of 1.24 (2–12°C) for tetanic force of single anterior tibialis fibers of *R. temporaria* (Edman, 1979).

From the above information, what force-calcium relation would be found experimentally using intracellular calcium indicators in an intact fiber? If the indicator is completely excluded from the intramyofibrillar space, the curve should be that in Fig. 2, using the upper abscissa. If the indicator is completely confined to the intramyofibrillar space, the

TABLE 2 Effect of temperature on the force-pCa relationship in skinned muscle fibers from the frog

Temperature (°C)	pH	Ca_{50} (μM)	N
22	7.18	1.45 (0.22, 6)*	3.66 (1.07, 6)
16	7.25	2.58 (0.42, 9)	4.85 (1.44, 9)
5	7.59	3.36 (0.56, 7)	5.29 (1.91, 7)

Data from each fiber were fitted by the Hill equation (Fig. 2). Means were calculated by averaging the individual Ca_{50} or N values obtained by fitting data from each fiber to the Hill equation.
* Standard deviation, number of observations.

[1] Note, however, that −0.4 mV is the difference between the predicted Donnan potential (−2.7 mV) and the average potential measured (−3.1 mV) in the absence of vanadate, consistent with the existence of a small diffusion potential.

[2] For example, a two-fold difference in free $[Mg^{2+}]$ (e.g., from 1 to 2 mM) will have a negligible effect on the Donnan potential (only 2 additional mequivalents per liter Mg^{2+} are available to titrate the ~42 mequivalents per liter fixed negative charge), whereas the same twofold difference will have a significant effect on the force-calcium relationship (Donaldson and Kerrick, 1975.)

curve should be that in Fig. 2, using the lower abscissa. Since commonly used indicators like aequorin, fura-2, indo-1, and fluo-3 enter both phases, they should give a curve between that determined by the upper and lower abscissas, i.e., essentially the same curve, because the two differ by only 0.09 log units.

DISCUSSION

Comparison of force-pCa curves with previous data in the literature

In the present study both temperature and solution pH were changed to represent the physiological case in intact frog muscle. In most other studies we know, pH was kept constant as temperature is varied. Although this makes comparison of the force-pCa data in Fig. 2 with previous work somewhat problematical, the data in Fig. 2 are in general agreement with other reports in the literature. Using mechanically skinned semitendinosus fibers from frog (*R. pipiens*), Godt and Lindley (1982) found that Ca_{50} and N were 1.63 μM and 3.81 (respectively) at 22°C, pH 7, and 1 mM Mg^{2+}. Similarly, Stevens and Godt (1990) report that Ca_{50} and N were 1.33 μM and 3.06 in a similar preparation under the same conditions. In all of these studies the experimental solutions were composed by using the same computer program to calculate solution composition; thus any variation in the values for Ca_{50} are not explained by differing constants for H^+, Ca^{2+}, and Mg^{2+} binding to EGTA. A rise in pH from 7 to 7.25 decreases the Ca_{50} by about 0.5 μM (Stevens and Godt, 1990). Therefore, the Ca_{50} at 22°C expected at pH 7.18 would be 1.0 to 1.3 μM (Stevens and Godt, 1990; and Godt and Lindley, 1982, respectively).

The Ca_{50} at room temperature under similar ionic conditions is in the same range in various vertebrate fast-twitch muscles. Goldman et al. (1987) find that Ca_{50} in skinned rabbit psoas fibers at 20°C and pH 7.1 is around 1.3 μM (pCa 5.9, estimated from their figure 4). Likewise, Stephenson and Williams (1985) report that Ca_{50} at 22°C and pH 7.1 in skinned rat extensor digitorum longus fibers is around 1.15 μM (pCa 5.94), and in twitch fibers from iliofibularis muscle of cane toad (*Bufo marinus*) it is 1.82 μM (pCa 5.74).

With respect to temperature effects, we find that the force-pCa curve steepens (i.e., N increases) as temperature is decreased, which is in agreement with other studies (Godt and Lindley, 1982; Stevens and Godt, 1990; Stephenson and Williams, 1985). There is a discrepancy in the literature, however, as to the effect of temperature on the positioning of the force-pCa curve. Some studies report that decreased temperature increases the calcium sensitivity of skinned skeletal muscle (frog: Godt and Lindley, 1982; rat fast-twitch and toad: Stephenson and Williams, 1985; canine cardiac: Fabiato, 1985; rabbit psoas: Goldman et al., 1987; frog: Stevens and Godt, 1990). Others report that calcium sensitivity decreases at lower temperature (cat cardiac: Brandt and Hibberd, 1976; cardiac: Harrison and Bers, 1989; rat cardiac and rabbit psoas; Sweitzer and Moss, 1990). Table 2 shows that, under our conditions, the calcium sensitivity of skinned frog semitendinosus fibers decreases significantly when temperature is decreased and pH is allowed to rise as it does in intact frog muscle. Because increasing pH at 22°C is known to *increase* calcium sensitivity (Robertson and Kerrick, 1979; Stevens and Godt, 1990), one would expect that the decrease in calcium sensitivity we observe would be even greater if pH was held constant.

Sweitzer and Moss (1990) suggest that if significant sarcomere shortening occurs during contraction, this could explain the apparent increase of calcium sensitivity at low temperature, because sarcomere uniformity is better under these conditions. Thus differences in end compliance may explain the discrepancies in the effect of temperature between the present result and that reported earlier (Godt and Lindley, 1982; Stevens and Godt, 1990). Although we did not routinely observe sarcomere uniformity during our present experiments, the method of attachment of fibers to our apparatus is improved over that used in earlier work from this laboratory (Godt and Lindley, 1982; Stevens and Godt, 1990). In the earlier work, fibers were clamped in small forceps tips, which led to considerable damage of this region of the preparation, and hence in extra compliance. In the present experiments, the fibers were wrapped around small stainless steel hooks attached to the apparatus (see Godt and Nosek, 1989, for details). This method probably causes less damage to the fiber in this region and better sarcomere uniformity throughout. The resolution of this issue is important, but it is beyond the scope of the current paper.

Relationship to intact fiber studies

Harkins et al. (1993), using fluo-3 in single fibers from *R. temporaria*, estimated that the level of myoplasmic Ca^{2+} at the threshold of activation of the myofilaments was 1.1–1.7 μM (pCa 5.95–5.77) at 16°C. Although the methods for calibration of fluorescent calcium indicators in skeletal muscle fibers are controversial (Westerblad and Allen, 1994; Baylor et al., 1994), our data at 16°C are in general agreement with this value; the threshold pCa value in Fig. 2 is between 6.0 (bath) and 6.09 (myofibrillar).

Allen et al. (1989) estimated the force-calcium relation in single, rested muscle fibers from the African frog *Xenopus* at 21°C using aequorin. Two examples of these relations are given in their figures 5 and 6. We manually extracted the data from these figures and fitted them to the Hill equation, yielding Ca_{50} and N values of 2.40 μM and 3.73 (their figure 5) or 2.96 μM and 2.95 (their figure 6). The slopes of these relations (N) are very similar to what we obtained in skinned *R. temporaria* fibers (3.66, Table 2). The Ca_{50} values are somewhat higher than what we obtained, however (1.45 μM, Table 2). Some of this apparent discrepancy may be due to the well-recognized weaknesses of the ae-

quorin calibration technique acknowledged by Allen et al. (1989), who suggest that their approach gives an indication rather than a precise calibration of $[Ca^{2+}]$. The discrepancy may be smaller than is apparent, however, because the intracellular pH of *Xenopus* muscle fibers under their conditions is probably lower than 7.0. Using ion-selective microelectrodes, Westerblad and Lannergren (1988) found that the mean intracellular pH of unfatigued *Xenopus* type 1 fibers was 6.93 and that of type 2 fibers was 6.99 in phosphate-buffered Ringer's at room temperature. In addition, Curtin (1987) found that the mean intracellular pH was 6.86 in both type 1 and type 2 fibers from *Xenopus*. Bathing solution pH in our experiments at room temperature was 7.18. Although Allen et al. (1989) did not identify which fiber types were used for the experiments in their figures 5 and 6, the intracellular pH in their fibers was lower than our case by at least 0.19 log units and could have been as much as 0.32 log units more acid. Inasmuch as acidification is known to decrease Ca^{2+} sensitivity, this would bring their estimates of Ca_{50} into closer agreement with ours.

We know of no experiments on the effects of pH on Ca^{2+} sensitivity of skinned *Xenopus* muscle fibers. However, in skinned fibers from *R. pipiens* at room temperature, Robertson and Kerrick (1979) found that decreasing pH from 7.5 to 7 decreased pCa_{50} to 0.7 μM in skinned *R. pipiens* fibers at 22°C. The changes in pCa_{50} for the smaller pH differences between the fibers of Allen et al. (1989) and ours will, of course, be smaller. Thus some discrepancy still exists, although in view of the acknowledged imprecision of the aequorin calibration, it is difficult to insist that the discrepancy is significant.

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