

# Calcium-Activated Tension of Skinned Muscle Fibers of the Frog

## *Dependence on Magnesium Adenosine Triphosphate Concentration*

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**ABSTRACT** The influence of MgATP on the  $\text{Ca}^{++}$ -activated isometric tension of skinned frog muscle fibers was examined in solutions containing:  $\text{Mg}^{++} = 5$  mM, creatine phosphate (CP) = 14.5 mM, creatinephosphokinase (CPK) = 1 mg/ml, total EGTA = 7 mM,  $\text{CaCl}_2$ , KCl, imidazole  $\geq 20$  mM so that ionic strength = 0.15, pH = 7.00, and MgATP = 2 mM, 0.1 mM, or 20  $\mu\text{M}$ . CP and CPK were necessary for these experiments as determined experimentally by their effect on the tension- $\text{Ca}^{++}$  relation, which was saturated for CP  $\geq 14.5$  mM. This was interpreted to mean that sufficient CP was present to effectively buffer MgATP intracellularly. Decreasing MgATP shifts the tension-pCa curve to higher pCa ( $-\log \text{Ca}^{++}$ ) so that, for half-maximal tension:  $\text{pCa}_{1/2} = 4.5$  for MgATP = 2 mM,  $\text{pCa}_{1/2} = 5.1$  for MgATP = 0.1 mM, and  $\text{pCa}_{1/2} = 5.8$  for MgATP = 20  $\mu\text{M}$ ; maximum isometric tension is the same in all cases, however. If MgATP was decreased to 1  $\mu\text{M}$ , tension at  $\text{Ca}^{++} > 10^{-8}$  M was 84% of the maximum  $\text{Ca}^{++}$ -activated tension in 2 mM MgATP and increased only slightly to 90% for pCa = 4.5. Weber (1970, *In The Physiology and Biochemistry of Muscle as Food*, Volume 2, E. J. Briskey, R. G. Cassens, and B. B. Marsh, University of Wisconsin Press, Madison, Wis.), using similar solutions, observed similar shifts in half-maximal calcium activation of rabbit myofibril ATPase rates. In explanation, Weber and Bremel (1971, *In Contractility of Muscle Cells and Related Processes*, R. J. Podolsky, editor, Prentice-Hall, Inc., Englewood Cliffs, N. J.; Bremel and Weber, 1972, *Nat. New Biol.*, **238**:97) have described a mechanism whereby, at low ATP, "rigor complexes" are formed between myosin and thin filament actin and, in turn, alter the calcium affinity of one class of the two  $\text{Ca}^{++}$ -binding sites on troponin, so that the thin filament is "turned on" for contraction at lower  $\text{Ca}^{++}$  levels. Tension data from skinned fibers substantially supports this hypothesis. A stability constant for CaEGTA of  $2.62 \times 10^{10} \text{ M}^{-1}$  was determined, with the help of F. N. Briggs, in solutions similar to those used for skinned fibers and was the same for 100 and 300 mM KCl.

## INTRODUCTION

It is widely held that adenosine triphosphate (ATP) is the energy substrate for muscle contraction (cf. Mommaerts, 1969). There is however considerable ignorance of exactly how at the molecular level ATP and the muscle proteins interact to produce contraction in the intact muscle. Under physiological conditions the intracellular concentration of ATP remains constant and contraction appears to be controlled by the intracellular level of ionized calcium (cf. Ebashi et al., 1969). On the other hand, Weber (1970), working with rabbit myofibrils, found that when the MgATP concentration was decreased from 2 mM to 10  $\mu$ M, the sigmoidal relationship between ATP hydrolysis and pCa ( $-\log \text{Ca}^{++}$ ) was shifted to lower calcium concentrations. From this and other observations, Weber and Bremel (1971; Bremel and Weber, 1972) propose a mechanism of cooperation among molecules of the thin filament whereby the formation of "rigor complexes" between thick filament myosin and thin filament actin (determined by MgATP bound to myosin) can affect the calcium affinity of the thin filament troponin molecules. At physiological levels of MgATP (millimolar range) there are practically no rigor complexes and contraction is activated only after calcium ions bind to both of two classes of regulatory sites of differing calcium affinity on troponin. At much lower MgATP (micromolar range) formation of rigor complexes causes cooperative transformation of the lower affinity troponin sites into high affinity sites so that contraction is initiated at lower free calcium concentrations.

To test this hypothesis on another system I examined the influence of MgATP concentration on the relationship between isometric tension and  $\text{Ca}^{++}$ . This is difficult to do properly on intact muscle cells due to their highly impermeable surface membrane, so I chose to work with skinned muscle fibers, that is, short lengths of muscle fibers whose membrane has been mechanically removed but whose internal elements are essentially intact (Costantin et al., 1965).

The concentration of MgATP in the interior of these skinned fibers will not be the same as that in the bathing medium because ATP is hydrolyzed by the contractile proteins and other cellular components. Simple calculations of inward diffusion of ATP into a cylinder with its simultaneous destruction (hydrolysis) inside (Hill, 1928) demonstrate that for probable hydrolysis rates there will be a gradient of ATP concentration across the cell diameter which would obscure the value of any experimental conclusions, especially at low levels of ATP. These diffusion problems can be overcome by inclusion of creatine phosphate (CP) and creatinephosphokinase (CPK) in the bathing

media to regenerate ATP from ADP at or near the hydrolytic sites. First off therefore it was necessary to determine the sufficient concentration of CP and then to proceed with experiments on the relation between MgATP and  $\text{Ca}^{++}$  activation.

## METHODS

### *Fiber Preparation*

Skinned twitch muscle fibers were prepared from the semitendinosus muscle of the common frog *Rana pipiens* using a procedure directly based on that of R. J. Podolsky (1968; Hellam and Podolsky, 1969). Both semitendinosus muscles were dissected out and placed in Ringer's solution of the following composition: NaCl, 117.5 mM; KCl, 2.5 mM;  $\text{CaCl}_2$ , 1.8 mM;  $\text{Na}_2\text{HPO}_4$ , 1.08 mM;  $\text{NaH}_2\text{PO}_4$ , 0.405 mM; pH  $\approx$  7.3; 10 mg/liter curare. Fibers from both heads of the muscle were used. To avoid tonic fibers, cells were taken only from the distal one-third of either head (Engel and Irwin, 1967).

The muscle was positioned with the distal tendon down and a small bundle (15–20 cells) a few millimeters long was taken using small scissors and jeweler's forceps. The bundle was laid on filter paper, pressed lightly with Kleenex to remove excess fluid and then placed in a Plexiglas dish with a glass bottom filled with water-saturated silicone oil (Dow Corning 200, 10 cs., Dow Corning Corp., Midland, Mich.), and observed with a Wild binocular dissecting microscope with a  $\times 0.5$  working distance extender (Wild Heerbrugg Instruments, Inc., Farmingdale, N. Y.). An undamaged fiber was selected and separated from the bundle with small stainless steel needles held in pin vices. Using these needles the fiber was "skinned", i.e. the sarcolemma, accompanying connective tissue, and some surface myofibrils were removed over a length of 3–4 mm. With the forceps, one end of the skinned portion was placed in the force transducer clamp; the other in a clamp attached to a micromanipulator. The skinned fiber section was then extended until just taut. The skinning and mounting procedures were carried out under oil.

### *Force Transducer*

The photoelectric force transducer used was based on the design of Hellam and Podolsky (1969) and was constructed and modified by Mr. Perry Johnson. It was exceptionally stable, rugged, and sensitive enough to measure tension from single skinned fibers. The fiber was held in a fiber clamp which is attached to a thick, inflexible wire onto which a vane is soldered. This vane partially interrupts the light path from a light-emitting diode (Texas Instruments, Inc., Digital Systems Div., Houston, Tex. TIL23) to a photodiode (Texas Instruments Co., IN2175). Tension in the fiber causes small movements of the vane which vary the amount of light hitting the photodiode, varying its resistance. This was detected as the output variation of a Wheatstone bridge, filtered to eliminate high frequency noise ( $\tau \approx 0.13$  s) and displayed on a Sanborn 7702A chart recorder (Hewlett Packard Co., Waltham Div., Waltham, Mass.).

The output of the transducer was linear up to 8.48 mN with a sensitivity of  $1.13 \times$

$10^3$  mV/mN. The natural period of the transducer-recorder system was 0.013 s. The practical minimum of detectable force was about  $4.9 \times 10^{-3}$  mN, and the overall compliance of the transducer was 18.3  $\mu\text{m}/\text{mN}$ .

#### *Solution Changer*

The various bathing solutions were contained in 5-ml wells milled into a Plexiglas block which was spring mounted on a platform, in a design similar to that of Hellam and Podolsky (1969). To change solutions the block was pressed down until the fiber clamps and fiber were clear of the well. The entire assembly was slid until the next solution well was beneath the clamps and the block released, immersing the fiber and clamps. This operation took but a few seconds. To prevent evaporation from the wells a piece of thin Plexiglas with a small handle was placed over the entire block when the solutions were standing idle.

#### *Bathing Solutions, Calculation of Solution Parameters*

Bathing solutions contained: total EGTA, 7 mM; imidazole,  $\geq 20$  mM;  $\text{MgCl}_2$  so that  $\text{Mg}^{++}$  was 5 mM, varying amounts of  $\text{CaCl}_2$ ,  $\text{Na}_2\text{ATP}$ ,  $\text{NaCP}$ , and CPK as indicated, and KCl so that  $\text{K}^+ + \text{Na}^+$  is 75 mM. Ionic strength ( $I/2$ ) was 0.15 and was kept constant in experiments with various concentrations of CP by changing the imidazole concentration but so that imidazole was 20 mM at the highest CP concentration. Imidazole was never decreased below 20 mM as Ogawa (1968) found that the Ca-EGTA binding constant increased as imidazole decreased below this level.

Calculation of the total concentrations of these ingredients needed to produce desired free concentrations of their various formation products involves solution of a number of simultaneous equations. A Raytheon PB 440 computer (Raytheon Data Systems Co., Norwood, Mass.) was used to solve the stability constant equations, enabling easy manipulation of selected ionic concentrations while holding others constant, utilizing a program written by Dr. Glenn Kerrick. The computer solved the binding equations using the stability constants in Table I.

#### *Constituents*

EGTA and imidazole were obtained from J. J. Baker Chemical Co., Phillipsburg, N. J.,  $\text{Na}_2\text{ATP}$ , disodium creatine phosphate (Sigma grade, 98–100% purity), and creatine phosphokinase<sup>1</sup> from Sigma Chemical Company, St. Louis, Mo.  $\text{MgCl}_2$ , KCl, HCl, and KOH (for pH adjustment) were reagent grade.  $\text{CaCl}_2$  was made by neutralizing reagent grade  $\text{Ca}(\text{OH})_2$  with HCl. Solution water was obtained from a Barnstead glass still.

The ATP stock solution was kept frozen. The other solutions were kept in a refrigerator. CP and CPK were kept in dry form in a desiccator in a freezer. The  $\text{MgCl}_2$  stock solution was made with a hitherto unopened bottle of the crystals and the resulting solution checked for  $[\text{Cl}^-]$  with a Cotlove chloridometer to eliminate uncertainties due to hydration of crystalline  $\text{MgCl}_2$ . The stocks were analyzed for Ca and Mg contamination with a Perkin-Elmer model 290B atomic absorption spectropho-

<sup>1</sup> Enzyme activity given as 50 units/mg, where one unit will transfer 1.0  $\mu\text{mol}$  of phosphate from CP to ADP per minute at pH = 7.4 at 30°C under Sigma assay conditions.

TABLE I  
STABILITY CONSTANTS USED

Ionic species	Stability constant	Source
	$M^{-1}$	
KATP <sup>-3</sup>	8	Botts et al. (1965)
CaATP <sup>-2</sup>	$2.5 \times 10^4$	Value between that of O'Sullivan and Perrin (1964) and of Taquikhan and Martell (1962), corrected for K <sup>+</sup> binding using data from Botts et al. (1965)
CaHATP <sup>-1</sup>	$3 \times 10^2$	As above
MgATP <sup>-2</sup>	$6 \times 10^4$	Value between that of O'Sullivan and Perrin (1964) (with temperature correction from Burton [1959]) and of Watanabe et al. (1963)
MgHATP <sup>-1</sup>	$6 \times 10^2$	Same as CaATP <sup>-2</sup>
HATP <sup>-3</sup>	$8.91 \times 10^6$	Smith and Alberty (1956)
H <sub>2</sub> ATP <sup>-2</sup>	$1.12 \times 10^4$	Martell and Schwartzbach (1956)
CaEGTA <sup>-2</sup>	$2.62 \times 10^{10}$	Experimental observation (see Appendix)
MgEGTA <sup>-2</sup>	$1.62 \times 10^5$	Schwartzbach et al. (1957)
HEGTA <sup>-3</sup>	$2.7 \times 10^9$	Chaberek and Martell (1959)
H <sub>2</sub> EGTA <sup>-2</sup>	$7.1 \times 10^8$	As above
H <sub>3</sub> EGTA <sup>-1</sup>	479	As above
H <sub>4</sub> EGTA	100	As above
CaHEGTA <sup>-1</sup>	$2.1 \times 10^5$	Schwartzbach and Senn (unpublished) in Sillén and Martell (1964)
MgHEGTA <sup>-1</sup>	$2.3 \times 10^3$	As above
MgCP	40	O'Sullivan and Perrin (1964)
H. Imidazole <sup>+</sup>	$1.23 \times 10^7$	Murphy and Koss (1968)

tometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Each sample for Ca determination contained 1% lanthanum and 5% HCl (vol/vol). All plastic- and glassware were washed with Sparkleen laboratory detergent (Fisher Scientific Co., Pittsburgh, Pa.) and rinsed thoroughly with tap water, followed by repeated rinsing with distilled water.

#### General Experimental Procedure

After the fiber was skinned and mounted in the transducer assembly it was transferred into "standard relaxing solution" containing 2 mM MgATP and 7 mM EGTA without added calcium (so that pCa > 8) and remained there for 1 min or longer until the tension level was constant. This level was thereafter used as the zero-tension reference. In most cases the fibers were then exposed to solutions of increasing calcium concentrations, allowing tension to rise to a steady level in each. Data obtained in this way were similar to but with less scatter than data obtained from fibers subjected to randomly ordered Ca<sup>++</sup> concentrations or to solutions of Ca<sup>++</sup> decreasing from a high concentration.

In experiments with MgATP other than 2 mM or with different CP concentrations the fibers were transferred from standard relaxing solution to a solution of pCa > 8 and with the desired MgATP or CP concentration. They remained there for at least 1 min before exposure to higher Ca<sup>++</sup> solution. If tension developed in solutions with

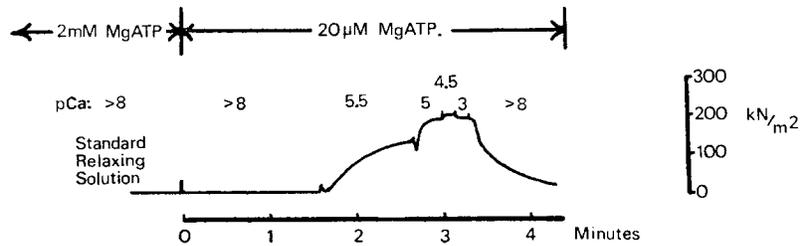


FIGURE 1. Tracing of typical experimental record of isometric tension of skinned fibers for various concentrations of  $\text{Ca}^{++}$ . Solution pCa shown above each portion of record. Tension scale at right shows calculated tension per cross-sectional area of fiber. Data from cell no. 3 (7 May 1971), 70  $\mu\text{m}$  diameter. Solutions contained 14.5 mM CP and 1 mg/ml CPK. Solution changes indicated by spikes on tension record, due to movement of fiber and transducer clamp through surface of bathing media.

pCa  $> 8$  it was allowed to reach a steady value before further solution changes were made. Fig. 1 illustrates a typical experimental record.

Experiments were performed at room temperature (22–25°C). During the course of each experiment the air temperature was regulated to within  $\pm 1^\circ\text{C}$  by air conditioning.

## RESULTS

### *Effect of CP and CPK on Isometric Tension*

As mentioned previously, the ATP level throughout the fiber cross section will not be uniform due to the ATPase of the cell components, which include sarcoplasmic reticulum and mitochondria as well as myofibrils. It is possible to calculate the nonuniformity by solving the diffusion equation for inward diffusion of ATP into a cylinder with simultaneous destruction (ATP hydrolysis) inside (see Discussion). These calculations show that an ATP regeneration system such as CP-CPK would be necessary, especially at the lower values of ATP.

The necessary level of CP under the experimental conditions was determined directly from the effect of addition of CP on the relation between  $\text{Ca}^{++}$  and isometric tension. This effect was examined at three levels of MgATP (2 mM, 0.1 mM, and 20  $\mu\text{M}$ ),  $\text{Mg}^{++}$  being 5 mM in all cases. CP in concentrations up to 19.3 mM, or in some cases, 24.1 mM, was added and the ionic strength of the solutions was held constant by compensatory changes in imidazole concentration. CPK was added to the bathing solutions to insure sufficiently high CP-ADP phosphotransferase activity to keep the ATP level constant. 1 mg/ml CPK was used and seemed sufficient; in a few cases 2 mg/ml was used without a discernible difference.

In solutions with 2 mM MgATP, addition of CP had no effect on tension in the relaxing solution (pCa  $> 8$ ) or the near-maximal tension produced by

pCa = 3.5. CP did however have a strong effect on the submaximal tension at pCa = 5.0, causing it to decrease markedly as CP increased to 14.5 mM. Increasing CP to 19.3 mM had no further effect. In Table II (see Discussion) this shift in the tension-Ca<sup>++</sup> relation can be seen by comparing the Ca<sup>++</sup> needed for half-maximal tension in the control (no CP) and 14.5 mM CP solutions.

In solutions with 0.1 mM or 20 μM MgATP but without CP there is no calcium sensitivity. That is to say, when a fiber was transferred from standard relaxing solution (2 mM MgATP) to a solution with pCa > 8 and with lower MgATP without CP, tension increased greatly and addition of high calcium (pCa 5.5, 5.0, or 3.5) caused no further tension increase. Similar loss of Ca<sup>++</sup> sensitivity was seen when fibers were transferred from solutions of pCa > 8 with 0.1 mM or 20 μM MgATP which contained CP to similar solutions which lacked CP.

In summary, experiments on the calcium-activated tension of skinned fibers seem to require addition of a soluble ATP-regeneration system such as CP-CPK since: (a) fibers in MgATP ≤ 0.1 mM absolutely require the presence of CP for calcium sensitivity, and (b) from diffusion calculations and from the observed shift of the submaximal Ca<sup>++</sup> activation curve, CP seems to be required even in 2 mM MgATP solutions. In all cases 14.5 mM CP seemed sufficient; increasing CP to 19.3 or 24.1 mM had no additional effect on Ca<sup>++</sup>-activated tension in any of the solutions.

#### *Effect on Tension-pCa Relation of Varying MgATP at Fixed Mg<sup>++</sup>*

Fig. 2 shows the relation between isometric tension and pCa in solutions with Mg<sup>++</sup> = 5 mM, imidazole = 20 mM, pH = 7.00, 14.5 mM CP with 1 mg/ml CPK,  $r/2 = 0.15$ , and three concentrations of MgATP: 2 mM, 0.1 mM, and 20 μM.

For the range of Ca<sup>++</sup> investigated, maximum tension was generated by pCa = 3.0 in 2 mM MgATP, pCa = 4.0 in 0.1 mM MgATP, and pCa = 4.5 in 20 μM MgATP. Data from fibers generating maximum tension less than 100 kN/m<sup>2</sup> were rejected. In Fig. 2 the tension generated in 2 mM MgATP solution with pCa = 3.0 was defined as 100% and represented an average tension of  $268.8 \pm 71.6$  kN/m<sup>2</sup> ( $\pm$  SD) assuming a circular fiber cross section.<sup>2</sup> Each fiber served as its own control since submaximal tension was expressed as a percent of the maximum in 2 mM MgATP, pCa = 3.0, in that particular fiber, and not as absolute tension. This was necessary as sarcomere length in each fiber was not observed and was somewhat different from fiber to fiber, leading to differences in the absolute value of maximum tension (Gordon et al., 1966; Hellam and Podolsky, 1969, Fig. 4; Schoenberg and Podolsky, 1972). In separate experiments sarcomere spacings were measured either

<sup>2</sup> Fiber diameters were determined in water-saturated silicone oil immediately after skinning.

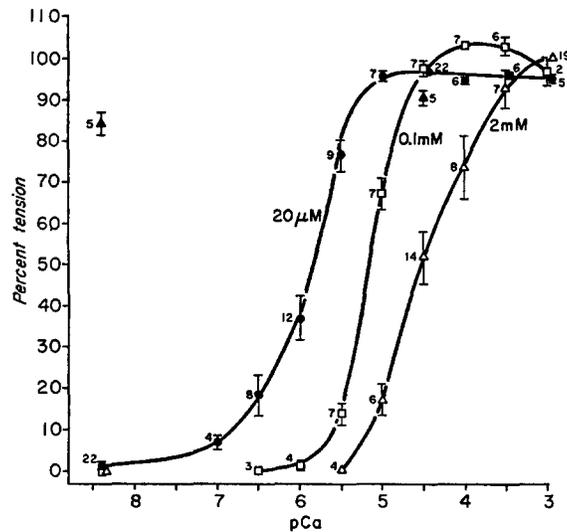


FIGURE 2. Total isometric tension of skinned fibers as a function of pCa. Tension in 2 mM MgATP, pCa 8, defined as 0% tension; tension in 2 mM MgATP, pCa = 3.0, defined as 100% tension. Concentrations by each curve refer to MgATP. All solutions contained 5 mM  $Mg^{++}$ , 14.5 mM CP and 1 mg/ml CPK (for other conditions, see text). Bars, or size of symbol, give standard error of the mean value for each pCa; small numbers by each point give number of experimental observations. A few symbols are slightly offset horizontally for legibility. Filled triangles refer to tension for 1  $\mu$ M MgATP. Curves fitted through data points by eye.

directly, with a microscope, or from the diffraction pattern produced by incident laser light. When the fibers were mounted and pulled just taut, as in these experiments, sarcomere spacings were 2.0–2.6  $\mu$ m, with most between 2.0 and 2.3  $\mu$ m. In this range, variations of sarcomere spacings should have only a small effect on the tension-pCa relation, although any such effect, if present, would increase scatter in the data only at very low pCa (Endo, 1972).

In Fig. 2, as MgATP is decreased, the tension-pCa curve shifts to lower  $Ca^{++}$ . If MgATP decreases too much, contractile protein interactions occur without calcium control, i.e. rigor complexes are formed. This can be seen in the experiments performed at 1  $\mu$ M MgATP (with 14.5 mM CP) in which tension in the relaxing solution increased greatly and an increase in calcium to pCa = 4.5 caused little increase in tension, i.e. calcium control is almost completely lost (see filled triangles in Fig. 2).

#### DISCUSSION

##### *Diffusion, Hydrolysis, and CP*

In these experiments it proved necessary to include CP and CPK in the bathing media. This was suggested from consideration of the kinetics of ATP

diffusion into the fiber with simultaneous ATP hydrolysis (by the ATPase of the cellular components) inside the cell and can be examined mathematically by solving the diffusion equation in cylindrical coordinates. If the ATPase rate per unit cell volume is constant one obtains in the steady state (Hill, 1928):

$$C = \frac{Kr_o^2}{4D} \left\{ \theta - (1 - \theta) \ln \left( \frac{1}{1 - \theta} \right) \right\},$$

where  $C$  = ATP concentration in the bathing medium;  $K$  = ATPase rate per unit cell volume;  $r_o$  = radius of fiber;  $D$  = diffusion constant for ATP in the cell;  $\theta$  = fraction of whole cross-sectional area in which ATP is present.

Infante et al. (1964) show that tension maintenance for a 1.5-s isometric tetanus in frog sartorius muscle at 0°C is associated with an ATPase rate ( $K$ ) of about  $0.8 \times 10^{-6}$  mol ATP/s g muscle. Assuming a  $Q_{10} = 2$  for this reaction (Bárány, 1967), at 20°C,  $K = 3.2 \times 10^{-6}$  mol ATP split/s g muscle (in agreement with data at 20°C of Canfield and Maréchal, 1973). If the radial diffusion coefficient ( $D$ ) for a skinned fiber is equal to the longitudinal diffusion coefficient,  $10^{-6}$  cm<sup>2</sup>/s at 20°C (Kushmerick and Podolsky, 1969) then, for a 100- $\mu$ m diameter fiber with a density of 1 g/cm<sup>3</sup>,  $\theta = 0.41$  when  $C = 2$  mM. That is, for 2 mM ATP outside the fiber, ATP is present in only 41% of the cell volume when the fiber is maximally activated with Ca<sup>++</sup>. For bathing medium concentrations of 0.1 mM ATP only 10% of the cell volume is "filled" with ATP under these conditions and for 20  $\mu$ M ATP less than 10% is filled. Thus at these ATP levels only a small volume of the fiber is filled with ATP and, moreover, even in the filled volume there is a radial gradient of ATP concentration. Addition of CP should, however, alleviate this problem and, from experiments with varying CP concentration, 14.5 mM CP seemed to be sufficient.<sup>3</sup> This value is comparable to the measured CP content of 24 mmol/kg muscle in intact frog phasic muscle (Carlson et al., 1967).

In the experiments of Infante et al. (1964) it is not clear how much of the ATP hydrolysis should be ascribed to tension maintenance and how much to Ca<sup>++</sup> release and sequestration, or to other processes. Nor is it clear that these ATPase activities are directly applicable to isometric tension maintenance in skinned fibers. Still further, it is well known (cf. Weber, 1970) that the ATPase rate of myofibrils decreases when ATP decreases below a certain level so that the " $K$ " in the diffusion equation is not really constant. It is nonetheless likely that an ATP regeneration system such as CP-CPK might be needed for skinned fibers for ATP concentrations around  $10^{-3}$  M and especially for concentrations around  $10^{-5}$  M. Furthermore, CP and CPK are normal intra-

<sup>3</sup> The diffusion of CP into the fibers should be at least as fast as that of ATP since the molecular weight of CP is but half that of ATP, so that, as a good approximation, the CP concentration can be added to that of ATP in the diffusion equation. In this case, for a 100- $\mu$ m fiber, over 90% of the cross-sectional area is filled with ATP even when the external ATP concentration is 20  $\mu$ M.

cellular constituents of intact muscle cells whose function, as in these experiments, is to keep intracellular ATP levels constant even when ATP hydrolysis rates are high. Thus skinned fiber bathing solutions containing CP in the concentrations used represent a better approximation to normal myoplasm than similar solutions without CP.

Addition of CP to the bathing solutions was absolutely necessary for experiments with MgATP concentrations of 0.1 mM or 20  $\mu$ M; without CP the fibers showed no calcium sensitivity. As has been demonstrated with skinned crayfish muscle fibers (Reuben et al., 1971; Brandt et al., 1972), experiments such as these with low MgATP can be performed without CP by ingeniously manipulating the bath concentrations of MgCl<sub>2</sub> and ATP. The intent of the present experiments, however, was to test the hypothesis of Weber and Bremel (1971; Bremel and Weber, 1972) which necessitated using solutions as similar to those of Weber (1970) as possible, i.e. 5 mM free Mg, with varying MgATP. Including CP in the bathing media greatly simplifies calculation and mixing of these solutions and insures homogeneity of Mg<sup>++</sup> and MgATP concentrations throughout the fiber cross section.

#### *Tension and Ca<sup>++</sup> at Physiological MgATP*

Table II presents all data in the literature for calcium-activated isometric tension of skinned frog muscle fibers at high (millimolar) ATP. It can be seen that, in the absence of CP, higher free Mg raises the calcium requirement for tension activation (see also Kerrick and Donaldson, 1972) and that, in all cases, the tension-Ca<sup>++</sup> curves are rather steep. In the presence of CP, the activation curve shifts even more to higher Ca<sup>++</sup> and is less steep.

These differences with CP can be easily explained according to the findings of Weber and Bremel (1971; Bremel and Weber, 1972). With no ATP-regenerating system, activation of tension and ATP hydrolysis by Ca<sup>++</sup> will greatly decrease the ATP level in the center of the fiber since ATP diffusion inward is relatively slow. Decreased ATP saturation of the myosin under these conditions gives rise to rigor complexes with thin filament actin which cooperatively alter the troponin low affinity Ca<sup>++</sup>-binding sites so that the thin filament is turned on at lower free calcium levels. Thus, in solutions with identical Mg<sup>++</sup> and MgATP, the activation threshold Ca<sup>++</sup> should be more or less the same with or without CP but, in the presence of CP (i.e. constant high MgATP throughout the fiber), the calcium required for half-maximal activation will be higher than when CP is not present. That this is the case can be seen by comparing the control (no CP) and the 14.5 mM CP curves for 5 mM Mg<sup>++</sup> and 2 mM MgATP in Table II.

(It should be noted that the curve in Fig. 2 for 2 mM MgATP is shifted far to the right (high Ca<sup>++</sup>) and probably represents an unphysiological case since it is unlikely that such high intracellular calcium levels are required for

TABLE II  
 $Ca^{++}$  AND TENSION OF FROG SKINNED MUSCLE FIBERS AT MILLIMOLAR ATP

Source	Solutions*		Threshold $Ca^{++}$	$Ca^{++}$ for half-maximum activation†	$Ca^{++}$ for maximum activation‡
	General	Mg & ATP			
Ebashi and Endo, 1968	$\Gamma/2=0.15$ pH=6.8§ T=4°C	Mg total 1 ATP total 4 <i>mM</i>	$1.3 \times 10^{-6}$ M	$4.0 \times 10^{-6}$ M	$3.2 \times 10^{-4}$ M
Hellam and Podolsky, 1969	$\Gamma/2 \cong 0.18$ (estimated) pH=7.0§ T=19-22°C	Mg total 1 ATP total 5	$\sim 3.2 \times 10^{-8}$ M ( $\sim 10^{-7}$ M corr.)¶	$1.8 \times 10^{-7}$ M ( $6.4 \times 10^{-7}$ M corr.)¶	$10^{-6}$ M ( $3.6 \times 10^{-6}$ M corr.)¶
Ebashi et al., 1969	$\Gamma/2=0.15$ pH=6.8 T=4°C	Mg total 4 ATP total 4	$1.3 \times 10^{-6}$ M	$3.2 \times 10^{-6}$ M	$10^{-4}$ M
As above	As above	Mg total 12 ATP total 4	$4.0 \times 10^{-6}$ M	$7.9 \times 10^{-6}$ M	$4.0 \times 10^{-4}$ M
Godt (unpublished observations)	$\Gamma/2=0.15$ pH=7.0§ T=22-24°C   Control, no CP	Mg <sup>++</sup> =5 Mg ATP = 2	$10^{-6}$ M	$2 \times 10^{-6}$ M	$10^{-6}$ M
Godt (Fig. 2, this paper)	$\Gamma/2=0.15$ pH=7.0§ T=22-25°C CP=14.5 mM	Mg <sup>++</sup> =5 Mg ATP=2	$3.2 \times 10^{-6}$ M	$3.2 \times 10^{-6}$ M	$10^{-3}$ M

\* For complete experimental conditions see references.

† Estimated from published curves.

§ Hellam and Podolsky (1969) observed no difference in tension-pCa curves in pH 6.5 and 7.0 solutions when influence of pH on apparent stability constant of CaEGTA taken into account. Similar results for pH 6.3 and 6.8 were reported by Endo (1967), thus small differences in pH here are insignificant.

|| Tension-pCa relation not affected by temperature change from 4 to 20°C. (Endo, 1967).

¶ Corrected for differences in CaEGTA binding constant used, see Ebashi and Endo (1968) and Appendix, this paper.

normal tension activation. It could be that the free Mg is too high in these experiments but, unfortunately, we have little idea of the physiological level of Mg<sup>++</sup> in the myoplasm for comparison. The rightward shift in the presence of CP could conceivably arise from a greatly increased calcium uptake by the sarcoplasmic reticulum due to contamination of the CP by inorganic pyrophosphate, as was inferred in the uptake experiments of Weber et al. [1966] on fragmented reticulum. This was ruled out, however, by thin layer chromatography of CP from the same lot as used in the present experiments, kindly performed by the Sigma Chemical Company laboratories. No pyrophosphate was detected [ $\ll 0.5\%$ ], the only impurities present being small amounts of free creatine and orthophosphate.)

#### *Tension Activation at Lower MgATP*

Weber's data (1970) on calcium activation of rabbit myofibrillar ATPase and the calcium activation of isometric tension in skinned frog muscle fibers (Fig. 2) can be directly compared since the bathing solutions are virtually identical. The only difference was the ionic strength, which in Weber's experiments is lower ( $I/2 = 0.09$ ) than for the present experiments ( $I/2 = 0.15$ ). This should shift the curves only slightly in view of the results of Portzehl et al. (1969) on myofibrillar ATPase. Table III demonstrates the different effects on Ca<sup>++</sup> sensitivity of decreasing MgATP in the two preparations.

It can be seen that for the three MgATP concentrations studied, rabbit myofibrils are more sensitive to Ca<sup>++</sup> than frog skinned fibers. This is probably not due solely to a species difference since Schaedler (1967) found, in glycerinated fibers from a variety of animals, that in the same fiber ATPase activation was always more sensitive to Ca<sup>++</sup> than was tension. In preliminary experiments using solutions identical to those used for Fig. 2, I find that the

TABLE III  
Ca<sup>++</sup> REQUIREMENT FOR ACTIVATION OF RABBIT MYOFIBRILS AND FROG SKINNED FIBERS

MgATP concentration	Preparation	Ca <sup>++</sup> for half-maximal activation*	Ca <sup>++</sup> for maximal activation
<i>mM</i>		<i>M</i>	<i>M</i>
2	Myofibrils	$6.3 \times 10^{-6}$	$10^{-4}$
	Skinned fibers	$3.2 \times 10^{-5}$	$10^{-3}$
0.1	Myofibrils	$3.2 \times 10^{-6}$	$2.0 \times 10^{-5}$
	Skinned fibers	$7.9 \times 10^{-6}$	$10^{-4}$
0.02	Myofibrils	$6.3 \times 10^{-7}$	$6.3 \times 10^{-6}$
	Skinned fibers	$1.6 \times 10^{-6}$	$3.2 \times 10^{-5}$

\* Data from Weber (1970) corrected for CaEGTA stability constant used in skinned fiber experiments (see Appendix).

ATPase activation of frog myofibrils is more sensitive to  $\text{Ca}^{++}$  than is the isometric tension of the skinned fibers and that the curve relating ATPase and pCa for frog myofibrils is almost identical to Weber's curve for rabbit myofibrils. (Some of this difference in activation threshold might also be due to the real difference in the physical state of the calcium-activated preparations since myofibrils shorten greatly during ATPase measurements while the skinned fibers in these experiments were kept at constant length.)

The major difference between my results and those of Weber (1970) is in the effect of MgATP on maximum ATPase rate and on maximum tension. Decreasing MgATP decreases the maximum  $\text{Ca}^{++}$ -activated ATPase rate of myofibrils, as would be expected from simple enzyme kinetics. In contrast, maximum  $\text{Ca}^{++}$ -activated tension of skinned fibers is virtually the same for MgATP of 2 mM or 20  $\mu\text{M}$  and is actually slightly higher for MgATP = 0.1 mM.

In both cases, however, decreasing MgATP shifts the activation curve to lower  $\text{Ca}^{++}$ , confirming the observations of Weber (1970). It is apparent, nonetheless, in the skinned fiber that the calcium activation mechanism and the amount of tension actually generated tend to respond differently as MgATP is decreased. Since the relationship at the molecular level between energy utilization and the mechanics of contraction is so poorly understood at the present time, it is difficult to interpret this difference.

In contrast to Weber and Bremel's hypothesis, Reuben et al. (1971) and Brandt et al. (1972) propose a contractile scheme, based on experiments at low MgATP in skinned crayfish muscles, whereby calcium antagonizes the "substrate inhibition" of MgATP. Brandt et al. (1972) show that increasing MgATP shifts the isometric tension-pCa curve to the right (their Fig. 2) as in my experiments. Also tension-pCa curves computed from their quantitative model (their Fig. 4) bear a striking resemblance to my experimental data (Fig. 2, this paper) in that large decreases in MgATP lead to a leftward shift of the curves without marked decrease of the maximal isometric tension possible. I have chosen to interpret the present data on skinned frog fibers in terms of Weber and Bremel, however, because of similarities in experimental conditions and because their experiments are more complete and direct than those of Reuben et al. (1971) and Brandt et al. (1972) who rely exclusively on measurements of isometric tension. (For an excellent review of control mechanisms in muscle contraction see Weber and Murray, 1973.)

#### APPENDIX

### CaEGTA Binding Constant

As the literature values for the CaEGTA stability constant vary widely it was necessary to determine this constant in solutions containing the constituents of skinned fiber

bathing solutions (Mg, ATP, and imidazole) and to investigate whether significant K-EGTA binding might occur. KCl is often used to increase ionic strength, so its effect on CaEGTA binding must be determined for proper buffering of free Ca<sup>++</sup>.

The following experiments were conducted with the assistance of Dr. F. Norman Briggs in his laboratory using a modification of the Chelex-partition method described by Briggs and Fleishman (1965). For complete details see Godt (1971).

**METHOD** To determine the binding constant of Ca to EGTA an insoluble Ca-chelator, Chelex-100 resin, was used to compete with EGTA for the calcium. In a solution containing KCl, Mg, ATP, EGTA, imidazole, and chelex, the calcium was partitioned into soluble (CaATP and CaEGTA) and insoluble (Ca-Chelex) fractions which were determined using a small amount of radioactive Ca<sup>45</sup> tracer added with the cold Ca<sup>40</sup>. Solutions contained: 5 mM MgCl<sub>2</sub>, 5 mM Na<sub>2</sub>ATP, 22.2 mM imidazole, 2 ml resin, 0.1 μC Ca<sup>45</sup>Cl<sub>2</sub>, 80 μM total EGTA, 100 or 300 mM KCl, varying CaCl<sub>2</sub>, pH = 7.0.

**REAGENTS** Na<sub>2</sub>ATP was obtained from P-L Biochemicals, Inc., Milwaukee, Wis., imidazole (Sigma grade 1) from Sigma Chemical Company, and Chelex-100 (200–400 mesh) from Calbiochem, San Diego, Calif. CaCl<sub>2</sub> was made by dissolving reagent grade CaCO<sub>3</sub> with concentrated HCl. MgCl<sub>2</sub> and KCl were reagent grade. Deionized water was used exclusively. Contaminating calcium in the stock solutions was determined with calcein in a Turner Fluorometer (G. K. Turner Associates, Palo Alto, Calif.) (Briggs and Fleishman, 1965).

The resin was prepared as follows: Chelex-100 was washed three times with 2 N HCl and then three times with deionized water. On the third wash the pH was adjusted to 7.0. It was then washed three times with 1 M MgCl<sub>2</sub> and on the third wash the pH was adjusted again to 7.0. After three more washings with deionized water it was brought up to a volume so that 4-ml stock = 1 ml packed volume.

#### *Results and Discussion*

Results are displayed in Fig. 3 as a Scatchard plot (Scatchard, 1949) of the ratio CaEGTA/Ca<sup>++</sup> versus CaEGTA for the two concentrations of KCl. The line was fitted to the points by eye. Since the line is not curved there is but one class of binding site for Ca as would be expected from the stereochemistry of EGTA. It is apparent that there is no effect on the Ca-EGTA binding of 100 mM ≤ KCl ≤ 300 mM.

The apparent stability constant (Eq. 1) was  $1.35 \times 10^6 \text{ M}^{-1}$  at pH = 7.0 in 5 mM MgCl<sub>2</sub>, 5 mM ATP, 22.2 mM imidazole, 100 mM ≤ KCl ≤ 300 mM. In skinned fiber solutions with Mg<sup>++</sup> < 10<sup>-2</sup> M, Ca<sup>++</sup> < 10<sup>-2</sup> M, and pH ≈ 7, the apparent stability constant:

$$K'_{\text{CaEGTA}} = \frac{\text{CaEGTA}^{-2}}{[\text{Ca}^{++}][\text{EGTA}_{\text{added}} - \text{CaEGTA}^{-2}]}, \quad (1 a)$$

and the "true" stability constant:

$$K_{\text{CaEGTA}} = \frac{\text{CaEGTA}^{-2}}{[\text{Ca}^{++}][\text{EGTA}^{-4}]}, \quad (2 a)$$

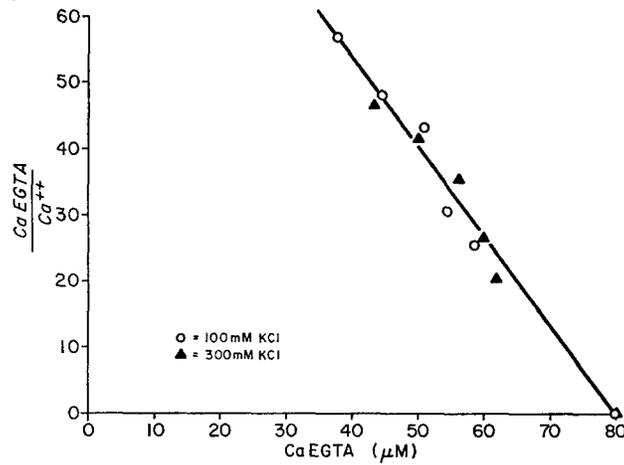


FIGURE 3. Scatchard plot of binding data for EGTA at pH = 7.00 with 100 and 300 mM KCl. See text for details.

are related as:

$$K_{CaEGTA} = K'_{CaEGTA}(1 + K_{HEGTA}[H^+] + K_{H_2EGTA}K_{HEGTA}[H^+]^2) \quad (3a)$$

(see Ringbom, 1963), so that:

$$K_{CaEGTA} = K'_{CaEGTA}(1.94 \times 10^4) \text{ at pH} = 7.0$$

and using the apparent stability constant from Fig. 3

$$K_{CaEGTA} = 2.62 \times 10^{10} M^{-1} = 10^{10.42} M^{-1}$$

As can be seen in Table IV this value compares favorably with other values in the literature determined in similar solutions, but differs from the widely used value obtained by Schwartzenbach et al. (1957). This difference may stem from the presence of imidazole which Ogawa (1968) has shown can cause substantial changes in the CaEGTA stability constant. Variation of KCl from 50 to 300 mM and the presence of MgCl<sub>2</sub> or ATP seem from Table IV to have little effect.

I wish to acknowledge the excellent help and encouragement of Professor A. M. Gordon and the helpful criticisms of the manuscript by Professor H. Portzehl. Thanks are also due the Sigma Chemical Co. for specially conducting the thin layer chromatography of the CP.

This work is based upon my thesis submitted to the University of Washington in partial fulfillment of the requirements for the Ph.D. degree.

This work was supported by grants from the National Institutes of Health (Training Grant GM 00260, NS 08384, NS 01752, and, for computer services, RR 00374).

Received for publication 6 September 1972.

TABLE IV

$K_{CaEGTA}^*$	KCl	Imidazole	MgCl <sub>2</sub>	ATP	pH	Method	Source and comments
$M^{-1}$							
$2.14 \times 10^{10}$ ( $10^{10.33}$ )	$mM$ 50	$mM$ 30	$mM$ 4	$mM$ 4	6.7	Chelex partition	Briggs and Fleishman (1965) (1 mM K oxalate also present)
$2.03 \times 10^{10}$ ( $10^{10.31}$ )	100	20	with or without	—	6.8	$Ca^{++}$ determined with murexide	Ogawa (1968)
$2.62 \times 10^{10}$ ( $10^{10.42}$ )	100 or 300	22.2	5	5	7.0	Chelex partition	Fig. 3
$9.33 \times 10^{10}$ ( $10^{10.97}$ )	100	—	—	—	Varying	pH-titration	Schwarzenbach et al. (1957)

\* Calculated for given pH using Eq. 3.

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