Characterization of the Effects of Mg^{2+} on Ca^{2+}- and Sr^{2+}-Activated Tension Generation of Skinned Skeletal Muscle Fibers

S. K. BOLITHO DONALDSON and W. GLENN L. KERRICK

From the Departments of Physiology and Biophysics, School of Medicine and the Department of Physiological Nursing, School of Nursing, University of Washington, Seattle, Washington 98195

ABSTRACT Changes in $[Mg^{2+}]$ in a millimolar range have a significant inverse effect on the Ca^{2+}- (or Sr^{2+}-)activated tension generation of skeletal muscle fibers. Single frog (*Rana pipiens*) *semitendinosus* muscle fibers were "skinned" (sarcolemma removed) and contracted isometrically in bathing solutions of varying $[Ca^{2+}]$ or $[Sr^{2+}]$ and $[Mg^{2+}]$ but at constant pH, $[MgATP^{2-}]$, $[K^+]$, $[CP^{2-}]$, [CPK], and ionic strength. Ca^{2+}- (or Sr^{2+}-)activated steady-state tensions were recorded for three $[Mg^{2+}]$'s: 5×10^{-5} M, 1×10^{-3} M, and 2×10^{-3} M; and these tensions were expressed as the percentages of maximum tension generation of the fibers for the same $[Mg^{2+}]$. Maximum tension was not affected by $[Mg^{2+}]$ within Ca^{2+}-activating or Sr^{2+}-activating sets of solutions; however, the submaximum Ca^{2+}- (or Sr^{2+}-)activated tension is strongly affected in an inverse fashion by increasing $[Mg^{2+}]$. Mg^{2+} behaves as a competitive inhibitor of Ca^{2+} and also affects the degree of cooperativity in the system. At $[Mg^{2+}] = 5 \times 10^{-5}$ M the shape of tension versus $[Ca^{2+}]$ (or $[Sr^{2+}]$) curve showed evidence of cooperativity of Ca^{2+} (or Sr^{2+}) binding or activation of the contractile system. As $[Mg^{2+}]$ increased, the apparent affinity for Ca^{2+} or Sr^{2+} and cooperativity of the contractile system declined. The effect on cooperativity suggests that as $[Mg^{2+}]$ decreases a threshold for Ca^{2+} activation appears.

INTRODUCTION

Mg^{2+} is important in muscle contraction but its exact role and mechanism of action have not been defined. The primary activator of muscle contraction is Ca^{2+} (Ebashi and Endo, 1968), but in order to generate Ca^{2+}-activated tension, both Mg^{2+} and ATP^{4-} must be present in solutions bathing the contractile proteins (Bendall, 1969). In skeletal muscle, Mg^{2+} is in a millimolar concentration range for both its total concentration (Conway, 1957; Walser, 1967; Bianchi, 1968) and its ionized, or free state (Günther, 1967; Günther and Dorn, 1971) when measured by a variety of techniques.

The finding that Mg concentration, total and free, is in a millimolar concentration range is significant in terms of understanding contraction in vivo since Mg^{2+} has been found to have a depressant action on standard measures of contraction when its concentration is increased in a millimolar range (Maruyama and Watanabe, 1962; Portzehl et al., 1969). Ebashi and Endo (1968) and Portzehl and co-workers (1969) found that the pCa-tension and pCa-ATPase relationships, respectively, were shifted in the direction of decreasing pCa[1] (increasing Ca^{2+} concentration) when Mg^{2+} concentration increased in a millimolar concentration range. In both of these studies, changes in $[ATP^{4-}]$ and $[MgATP^{2-}]$ were being made simultaneously with changes in $[Mg^{2+}]$ making it impossible to attribute the effects exclusively to $[Mg^{2+}]$ changes. However, Kerrick and Donaldson (1972), using skinned (sarcolemma removed) frog skeletal muscle fibers and measuring submaximum and maximum isometric tension generation, were able to separate the effects of changes in $[MgATP^{2-}]$ and $[ATP^{4-}]$ from those of Mg^{2+} and found them to be very small in comparison to the effect of changes of $[Mg^{2+}]$ in a millimolar concentration range. Thus $[Mg^{2+}]$ by itself was found to have a significant inverse effect on submaximum Ca^{2+}-activated tension generation in frog skeletal muscle fibers.

A question remains, however, as to the exact nature of this inverse effect of Mg^{2+} on Ca^{2+}-activated tension generation. Mg^{2+} binds in the system but the exact number and location of sites are not known. It has been shown that Mg^{2+} binds to a system of proteins which, because of contamination, probably contained troponin and tropomyosin in addition to F-actin and G-actin (Martonosi et al., 1964). There is evidence that Mg^{2+} interacts with troponin since when present in solution with troponin, Mg^{2+} changes troponin's electrophoretic mobility (Chowrashi and Kaldor, 1970) and appears to bring about a change in its conformation (Kawasaki and van Eerd, 1972; Gruda et al., 1973). Furthermore, increasing Mg^{2+} concentration in a millimolar range was associated with decreased binding of Ca^{2+} to myofibrils at a given Ca^{2+} concentration (Weber et al., 1969) and a decreased Ca^{2+}-induced conformational change in the Ca^{2+} binding component of troponin at a given Ca^{2+} concentration (Kawasaki and van Eerd, 1972). In studies of isolated troponin, Mg^{2+} appears to compete with Ca^{2+} for some of the binding sites (Potter and Gergely, 1974), but the relationship of these sites to tension generation is not known.

In addition Mg^{2+} has been shown to affect the binding of Ca^{2+} to myosin (Bremel and Weber, 1975). However, the relationship of cation binding to these sites and tension generation is also not known.

The purpose of this study was to elucidate the nature of the interaction of

[1] $pCa = -\log_{10}[Ca^{2+}]$.

Mg^{2+} and Ca^{2+} in Ca^{2+}-activated tension generation in single skinned (sarcolemma removed) frog skeletal muscle fibers. Both Ca^{2+} and its analogue Sr^{2+} were used to activate contraction, and the effects of changing $[Mg^{2+}]$ on the Ca^{2+}-activated and Sr^{2+}-activated isometric tensions were studied. Data for complete pSr^2 versus isometric tension and pCa versus isometric tension relationships were obtained at each of three Mg^{2+} concentrations (free): 5×10^{-5}, 1×10^{-3}, and 2×10^{-3} M. This duality allows the effect of Mg^{2+} to be classified since Sr^{2+} is an analogue of Ca^{2+} in activating tension generation and it has a lesser affinity for the system, as demonstrated in preliminary work in this laboratory. Thus the effects of Mg^{2+} on Sr^{2+}-activated tension relative to the effects on Ca^{2+}-activated tension generation could exclude some and make other mechanisms of its action more likely.

METHODS

Preparation

Single twitch fibers from the *semitendinosus* muscles of frogs (*Rana pipiens*) were used. A bundle of 10 to 20 fibers was cut from the distal end of the dorsal head of the semitendinosus (immersed in Ringer's solution) in order to avoid tonic fibers (Engle and Irwin, 1967). After being blotted dry the fiber bundle was then placed in a glass-slide-bottomed dish containing silicone oil (Dow Corning 200, 10 cs, Dow Corning Corp., Midland, Mich.). Visualization was accomplished using back lighting and a Wild binocular dissecting microscope (Wild Heerbrugg Instruments Inc., Farmingdale, N. Y.) with a × 0.5 working distance extender.

In the silicone oil, single fibers were separated from the bundle. To remove the membranous diffusion barrier, single fibers were skinned (their sarcolemma peeled off) (Natori, 1954; Podolsky, 1968) in a relaxing solution injected into the silicone oil. The relaxing solution had the following composition: pCa = 9-8, pMg = 4.3, 7 mM EGTA, 70 mM K^+, 76 mM Cl^-, 3.6 mM adenosine triphosphate (ATP), 15 mM creatine phosphate (CP^{2-}), 2 mM $MgATP^{2-}$, and imidazole (pH = 7.0 and ionic strength = 0.15 M). The diameter of each fiber was measured with the microscope graticule immediately after skinning. This diameter measurement might be approximately 10% larger than the true (unskinned) diameter due to a slight swelling of the skinned fibers at rest length in a relaxing solution (Matsubara and Elliott, 1972).

Isometric Tension Measurement

The skinned fibers were then mounted in the forcep tips of a photoelectric force transducer which was similar to that used by Hellam and Podolsky (1969). The compliance of the transducer was 0.714 μM/mg and the shortening of the fibers was always less than 5% of the mounted length of 2–3 mm since the maximum force measured was 110 mg (corresponding to a maximum fiber diameter of 80 μm). The sensitivity of the transducer was 35 V/G_m of force and voltage output linear to well over a G_m.

[2] $pSr = -\log_{10}[Sr^{2+}]$.

A base-line voltage was obtained with the mounted fiber immersed in a relaxing solution. The fibers were not stretched beyond rest length during mounting or skinning since Endo (1973) found that activation of skinned skeletal muscle fibers for a given Ca^{2+} concentration increases with increasing fiber length beyond rest or slack length. As evidence that the fibers in this study were not mounted under tension and did not generate tension in the relaxing solution, when fibers broke the voltage dropped within a few millivolts (<0.7 mg) of the original base-line voltage for each fiber when it was intact (i.e., to a very small percentage of the force generated during maximum tension generation).

Bathing Solutions

The fibers were dipped into 4-ml baths of electrolyte solutions covered with silicone oil to prevent evaporation. Room temperature was maintained at 20 ± 1°C. All bathing solutions contained 7 mM EGTA, 70 mM K^+, ATP^{4-}, 15 mM CP^{2-}, 2 mM $MgATP^{2-}$, Cl^-, 15 U/ml creatine phosphokinase (CPK) per milliliter of bathing solution, imidazole (pH = 7.0), and glass distilled water. Ionic strength was maintained at 0.15 M for each solution. Three Mg^{2+} concentrations were used: 5×10^{-5}, 1×10^{-3}, and 2×10^{-3} M. At each $[Mg^{2+}]$ two sets of solutions were mixed: (*a*) a set containing variable concentrations of Ca^{2+} (pCa's ranging from 9 to 8,[3] or no added Ca^{2+}, up to pCa = 3.1 for maximum tension generation at $Mg^{2+} = 2 \times 10^{-3}$ M) and (*b*) a set containing variable Sr^{2+} concentrations (no added Sr^{2+}[3] up to pSr = 3.0 for maximum tension generation at $Mg^{2+} = 2 \times 10^{-3}$ M). However, Sr^{2+} and Ca^{2+} were never both added to any solution. Thus separate and complete pCa and pSr versus isometric tension curves were obtained at each Mg^{2+} concentration.

$MgATP^{2-}$ concentration was held constant (2 mM, according to binding constants used) and $[ATP^{4-}]$ was allowed to vary as $[Mg^{2+}]$ was increased. In both the Sr^{2+} and Ca^{2+} solutions $[ATP^{4-}]$ varied from 6.6×10^{-4} to 1.7×10^{-5} M as $[Mg^{2+}]$ was increased from 5×10^{-5} to 2×10^{-3} M. The variations in $[ATP^{4-}]$ should not affect the results since it has been established that variations in $[MgATP^{2-}]$, $[ATP^{4-}]$, and $[CaATP^{2-}]$ in the concentration ranges occurring in this study do not significantly alter the changes seen in submaximum Ca^{2+}-activated tension associated with changes in $[Mg^{2+}]$ (Kerrick and Donaldson, 1972). Furthermore, the substrate for the enzyme myosin is believed to be $MgATP^{2-}$ (Eisenberg and Moos, 1970) and $MgATP^{2-}$ binding to myosin is approximately maximum at 2.0 mM $[MgATP^{2-}]$ (Maruyama and Weber, 1972).

In order to ensure that the system would not be ATP limited and that gradients in $MgATP^{2-}$ concentration within the fiber would not occur, an ATP regenerating system of CP and CPK was used. This particular regenerating system was selected because it is the one which is present in frog muscles. Frog phasic muscles contain 3 mM total ATP/kg of muscle (Murphy, 1966; Mommaerts and Wallner, 1967) and 24 mM CP/kg of muscle (Conway, 1957; Carlson et al., 1967). The CPK and CP^{2-} concentrations were maintained at constant values of 15 U/ml and 15 mM, respectively, in all bathing solutions. Doubling the CPK concentration did not alter the data at any Mg^{2+} concentration.

[3] All relaxing solutions contained some Ca^{2+} due to contamination.

It has been demonstrated that the $CaEGTA^{2-}$ binding constant is dependent upon imidazole concentration in a range below 0.02 M (Ogawa, 1968), but in this study imidazole concentration was always greater than 0.02 M (75.3–83.2 mM for Ca^{2+} solutions). The ionized imidazole concentration was varied slightly to keep ionic strength of all of the solutions constant at 0.15 M but was almost identical in solutions which resulted in generation of comparable tension levels at the highest and lowest $[Mg^{2+}]$'s.

The imidazole concentration variations at each $[Mg^{2+}]$ were less than 5 mM for either Sr^{2+} or Ca^{2+}, but the Sr^{2+} solutions had more imidazole because, although the relaxing solutions were essentially identical for Ca^{2+} and Sr^{2+}, the basic ionic strength of the Ca^{2+} contracting solutions (ionic strength calculated before the addition of imidazole at pH = 7.0) was approximately 0.015 M greater than the basic ionic strength for comparable (same $[Mg^{2+}]$ and causing the same percentage of maximum tension generation) Sr^{2+} contracting solutions. This difference occurs primarily due to differences in the concentrations of the EGTA complexes.

Ionic strength of the solutions was maintained at a constant value by adjusting the imidazole concentration. In order to make sure that this did not affect the results, pCa versus tension data for pMg = 4.3 and 3.0 were also collected in solutions containing 100 mM K^+ and μ = 0.15 M (imidazole decreased by 33.3 mM). These two pMg's were selected because the effect of Mg^{2+} on the position and steepness of the pCa versus tension relationship is affected to the greatest extent in this range of Mg^{2+} concentration. K^+ concentration was increased to better approximate that of intracellular fluid; and to maintain ionic strength at 0.15 M, imidazole concentration was decreased by more than one-third. Individual fibers were tested in solutions having identical Ca^{2+} concentrations but either 70 or 100 mM K^+. There was no statistically significant difference between these paired data at 70 and 100 mM K^+. Therefore, neither the K^+ or imidazole concentrations seemed to affect the data over a broad range of concentrations.

The ionic equilibrium for each solution, buffer capacity, and the ionic strength were calculated by digital computer using binding constants from the literature and a program written by Kerrick (Kerrick and Donaldson, 1972). The binding constants are shown in Table I. For each solution the desired ionic strength and ionic concentrations of K^+, Mg^{2+}, Ca^{2+} or Sr^{2+}, H^+, $MgATP^{2-}$, CP^{2-}, and the total concentration of K_2EGTA (EGTA titrated to pH = 8.0 with KOH) were specified and the computer calculated the total amount of $MgCl_2$, $CaCl_2$ or $SrCl_2$, KCl, Na_2ATP, Na_2CP, and imidazole to be added. The amount of Na^+ added as Na_2ATP and Na_2CP was treated as K^+ because Na^+ and K^+ have similar binding constants for ATP^{4-} (O'Sullivan and Perrin, 1964). After the addition of the calculated amounts of these salts to double-distilled water, the pH of the solution was measured and titrated with HCl to pH = 7.0. The solutions were immediately frozen in small vials and only the amount required for a given experiment was thawed just before data collection (storage was less than 2 mo). CPK was added after the solutions were thawed.

As a check on the accuracy of the binding constants, the computer also predicted the amount of HCl which should be required to bring each solution to pH = 7.0. It was consistently found, in comparing the predicted to the actual amount of HCl added to titrate to pH = 7.0, that the error was less than a few percent. However,

TABLE I

BINDING CONSTANTS USED IN THE COMPUTER PROGRAM

Definition of binding constant	Value	Reference
	M^{-1}	
$[CaATP^{2-}]/[Ca^{2+}]\ [ATP^{4-}]$	2.5×10^4	Values chosen between those of O'Sullivan and Perrin (1964) and Taqui Kahn and Martell (1962) and corrected for K^+ binding using data from Botts et al. (1965).
$[CaHATP^{-}]/[Ca^{2+}]\ [HATP^{3-}]$	3×10^2	
$[SrATP^{2-}]/[Sr^{2+}]\ [ATP^{4-}]$	7.95×10^3	
$[SrHATP^{-}]/[Sr^{2+}]\ [HATP^{?-}]$	2.7×10^2	
$[CaEGTA^{2-}]/[Ca^{2+}]\ [EGTA^{4-}]$	2.6×10^{10}	Observation of Robert E. Godt done on similar solutions in the laboratory of F. N. Briggs (Godt, 1974).
$[CaHEGTA^{-}]/[Ca^{2+}]\ [HEGTA^{3-}]$	2.1×10^5	Schwarzenbach and Senn (unpublished) in Sillen and Martell (1964).
$[SrEGTA^{2-}]/[Sr^{2+}]\ [EGTA^{4-}]$	3.2×10^8	
$[SrHEGTA^{-}]/[Sr^{2+}]\ [HEGTA^{3-}]$	2.3×10^4	
$[MgATP^{2-}]/[MG^{2+}]\ [ATP^{4-}]$	6×10^4	Value selected between that of Watanabe et al. (1963) and that of O'Sullivan and Perrin (1964) with temperature correction from Burton (1959).
$[MgHATP^{-}]/[Mg^{2+}]\ [HATP^{3-}]$	6×10^2	Same as $[CaATP^{2-}]/[Ca^{2+}]\ [ATP^{4-}]$.
$[MgEGTA^{2-}]/[Mg^{2+}]\ [EGTA^{4-}]$	1.62×10^5	Schwarzenbach et al. (1957).
$[MgHEGTA^{-}]/[Mg^{2+}]\ [HEGTA^{3-}]$	2.3×10^3	Same as $[CaHEGTA^{-}]/[Ca^{2+}]\ [HEGTA^{3-}]$
$[KATP^{3-}]/[K^{+}]\ [ATP^{4-}]$	8	Botts et al. (1965).
$[HATP^{3-}]/[H^{+}]\ [ATP^{4-}]$	8.9×10^6	Smith and Alberty (1956).
$[H_2ATP^{2-}]/[H^{+}]\ [HATP^{3-}]$	1.1×10^4	Martell and Schwarzenbach (1956).
$[HEGTA^{3-}]/[H^{+}]\ [EGTA^{4-}]$	2.7×10^9	Chaberek and Martell (1959).
$[H_2EGTA^{2-}]/[H^{+}]\ [HEGTA^{3-}]$	7.1×10^8	
$[H_3EGTA^{-}]/[H^{+}]\ [H_2EGTA^{2-}]$	480	
$[MgCP]/[Mg^{2+}]\ [CP^{2-}]$	40	O'Sullivan and Perrin (1964).
$[SrCP]/[Sr^{2+}]\ [CP^{2-}]$	20	Smith and Alberty (1956) corrected to correspond with $[MgCP]/[Mg^{2+}]\ [CP^{2-}]$.
$[CaCP]/[Ca^{2+}]\ [CP^{2-}]$	20	
$[HCP^{-}]/[H^{+}]\ [CP^{2-}]$	500	Perrin (1965).
$[H_2CP]/[H^{+}]\ [HCP^{-}]$	3.8×10^4	

this type of check would not be very sensitive to certain errors (e.g., in K_{MgCP} which is discussed later).

To ensure accuracy of mixing, several checks were employed. Total Cl and ATP concentrations of all appropriate stock solutions were measured. In addition all sets (graded increasing pCa or pSr at a fixed $[Mg^{2+}]$) of solutions were mixed at least twice to make sure that the data were comparable from batch to batch. Another safeguard used was to omit one or two solutions of intermediate pCa (or pSr) from a particular set of solutions with graded pCa (or pSr) and to mix them separately at another time. Experiments were done to make sure that these separately mixed solutions gave the usual graded amount of tension when used in combination with the other solutions of that particular set.

Protocol for Data Collection

In all instances the steady-state change in voltage output of the transducer was used as the measure of tension generated. Base line was determined by drawing a straight line joining the ends of the tails of the relaxation phase of the tension-time recording.

In the following discussion "submaximum $[Ca^{2+}]$ (or $[Sr^{2+}]$)" and "maximum $[Ca^{2+}]$ (or $[Sr^{2+}]$)" refer to cation concentration at which the tension generation is submaximum or maximum, respectively. All fibers included generated an initial maximum tension of at least 1-kg/cm^2 cross-sectional area and were discarded when maximum tension declined to 50 % of the initial value. There was a slight decline in tension generation with each contraction which was much less in this study where CP and CPK were included in the bathing solutions as compared with the rate of decline of tension seen without the ATP-regenerating system (Kerrick and Donaldson, 1972). Maximum $[Ca^{2+}]$ (or $[Sr^{2+}]$) at each $[Mg^{2+}]$ was determined by using a stepping protocol which will be discussed later. Ca^{2+}- or (Sr^{2+}-)activated tensions at each $[Mg^{2+}]$ reached a plateau as $[Ca^{2+}]$ (or $[Sr^{2+}]$) was increased (plateau range = 0.4–0.6 pCa or pSr units) and then began to decline as the divalent cation concentration increased further. The minimum $[Ca^{2+}]$ (or $[Sr^{2+}]$) needed to reach the plateau tension at each $[Mg^{2+}]$ was used to measure maximum tension generation. There was no shift in base-line tension as a function of $[Mg^{2+}]$ nor was there any difference in base line between Ca^{2+} and Sr^{2+} solutions at a fixed $[Mg^{2+}]$, as would be expected since the pSr and pCa = eight to nine solutions at each $[Mg^{2+}]$ were identical. Two basic protocols were used in data collection.

One protocol incorporated bracketing of a test maximum contraction in one set of solutions with a maximum contraction from another set. The bracketing technique was used in this study primarily to check the maximum Ca^{2+}- (or Sr^{2+}-)activated tensions at various $[Mg^{2+}]$'s relative to each other and also to compare the maximum Ca^{2+}-activated tension at a given $[Mg^{2+}]$ to the maximum Sr^{2+}-activated tesnsion at the same $[Mg^{2+}]$. Each of the three contractions required for a single datum consisted of (*a*) equilibrating the fiber in a relaxing solution (pCa or pSr = 8–9, that is with no added Ca^{2+} or Sr^{2+}) at a specific $[Mg^{2+}]$; (*b*) then contracting the fiber by dipping it into a solution at the same $[Mg^{2+}]$ and a maximum $[Ca^{2+}]$ or $[Sr^{2+}]$; and (*c*) finally relaxing the fiber in a relaxing solution at the same $[Mg^{2+}]$.

Fig. 1 illustrates the protocol used in the comparing of Ca^{2+}-activated tensions

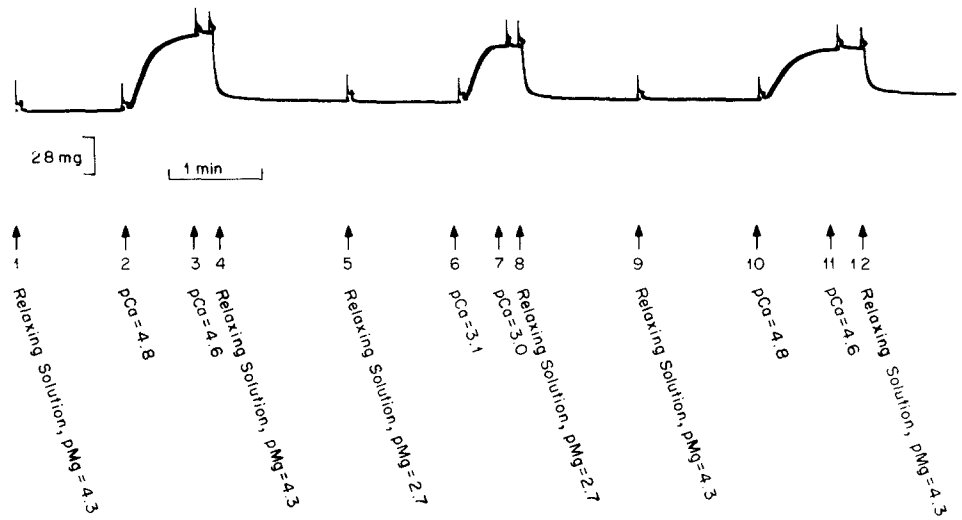

FIGURE 1. Maximum $[Ca^{2+}]$ contractions at two Mg^{2+} concentrations. Arrows indicate times at which changes in the bathing solution were made. Changes are indicated below the arrow number. See text for details. Tension increases upwards and the abscissa shows time. Fiber diameter = 60 μm. $[Mg^{2+}] = 5 \times 10^{-5}$ M for arrows 1–4 and 9–12. $[Mg^{2+}] = 2 \times 10^{-3}$ M for arrows 5–8.

generated at $[Mg^{2+}] = 5 \times 10^{-5}$ M and 2×10^{-3} M. Here the test is the maximum contraction at pMg = 2.7 and the contractions bracketing it are maximum ones at pMg = 4.3. To obtain the percentage of maximum tension generation the ratio of the test contraction divided by the average of the bracketing contractions is multiplied by 100. Thus the middle tension (test contraction) in Fig. 1 is normalized to a maximum tension obtained by averaging the values for the first and third maximum Ca^{2+}-activated tensions (bracketing contractions).

The stepping protocol, which was used primarily to obtain percentages of maximum tension generation for submaximum $[Ca^{2+}]$ and $[Sr^{2+}]$, differed from the bracketing one in that the fiber was not relaxed after each contraction. Instead the fiber was, after initial equilibration in a relaxing solution (no Ca^{2+} or Sr^{2+} added), contracted at a submaximum $[Ca^{2+}]$ (or $[Sr^{2+}]$) and then at maximum $[Ca^{2+}]$ (or $[Sr^{2+}]$) at that $[Mg^{2+}]$ (thus the tension "steps" from submaximum to maximum) before again relaxing it. Fig. 2 illustrates the stepping technique. In part B steady-state tensions were achieved and thus the submaximum tensions could be divided by the corresponding maximum tensions generated as a fiber was stepped from submaximum $[Ca^{2+}]$'s (arrows 8, 11, 14) to maximum $[Ca^{2+}]$'s (arrows 9, 12, 15). The ratio was multiplied by 100 to obtain percentage of maximum tension. Note also in Fig. 2 the graded decline in rate of the rise of tension and steady-state submaximum tension generation as $[Ca^{2+}]$ is decreased. It had already been verified that maximum $[Ca^{2+}]$ was pCa = 3.0 for the fiber in Fig. 2. The verification procedure is shown in Fig. 1 where the fiber was stepped from a solution containing pCa = 4.8 at arrow 2 to one containing pCa = 4.6 at arrow 3 to make sure that a plateau in steady-state tension had been achieved.

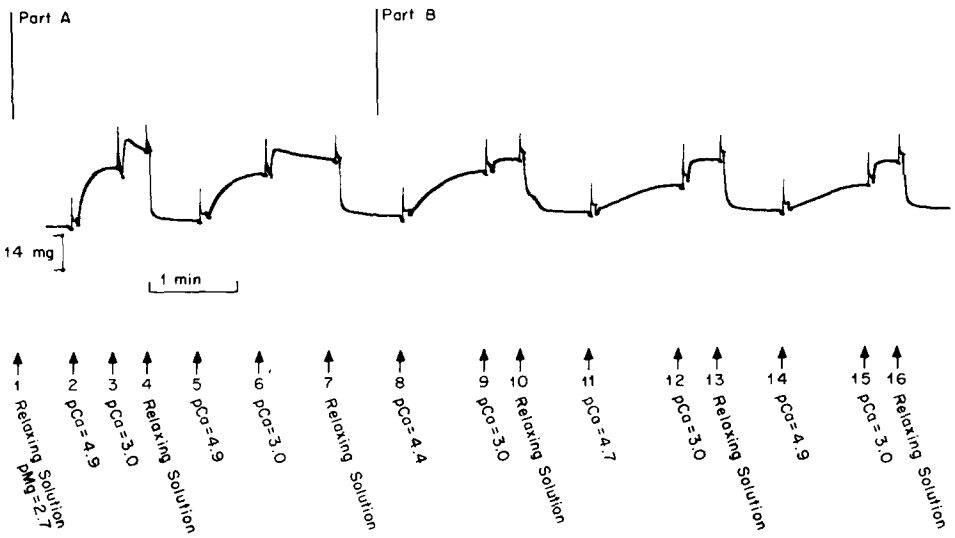

FIGURE 2. Submaximum and maximum [Ca^{2+}] contractions according to the stepping protocol. Arrows indicate times at which changes in the bathing solution were made. Changes are indicated below the arrow number. See text for details. Tension increases upwards and the abscissa shows time. Fiber diameter = 50 μm. In all solutions [Mg^{2+}] = 2×10^{-3} M.

Fig. 2 A, illustrates that often a steady-state tension is not achieved immediately. Initial tension transients in the maximum (and less frequently in the submaximum) Ca^{2+} and Sr^{2+} solutions were seen more often during stepping than bracketing. It was therefore important that only steady-state tensions be used, and therefore, contractions were maintained at least 15–20 s beyond the achievement of a plateau in tension. For a given submaximum concentration there was no statistically significant difference between the means of the percentages of maximum tensions obtained with each protocol. Also there was no consistent variation in the raw data or means of the percentages of maximum tension generation related to a particular protocol.

The order of variations in Ca^{2+}, Mg^{2+}, and Sr^{2+} concentrations were randomized for each fiber and between fibers. Earlier contractions were repeated at the end of a sequence to verify reliability. Individual fibers were tested at the same Ca^{2+} or Sr^{2+} concentrations at all Mg^{2+} concentrations as well as different Ca^{2+} or Sr^{2+} concentrations at a given level of Mg^{2+}, although it was not possible to collect a complete set of data for any one fiber.

For the statistical analysis each percentage of maximum tension value was treated as a datum. All of the data for a given Ca^{2+} and Mg^{2+} concentration were averaged so that the means could be plotted and compared. Data from a maximum of two fibers were collected at each Ca^{2+} concentration.

RESULTS

The technique of data collection yielded data that are very similar from fiber to fiber and for multiple contractions of any one fiber even though absolute

tension generations declined gradually with time. One fiber was contracted in Sr^{2+} solutions 17 times and another fiber was contracted 24 times in Ca^{2+} solutions and yet the data obtained for each submaximum $[Sr^{2+}]$ and $[Ca^{2+}]$, respectively, are very similar (no statistically significant difference at 0.05 confidence level) from early to later contractions where only steady-state tension generations are used. The fact that the fibers were still generating more than 50% of their original tension (which was greater than 1 kg/cm^2 cross-sectional area) is attributed to maintenance of constant $[ATP^{4-}]$ and $[MgATP^{2-}]$ via the CP and CPK regenerating system. Fibers contracted in similar bathing solutions without the addition of CP and CPK deteriorated (in terms of a decline in maximum tension generation) far more rapidly (Kerrick and Donaldson, 1972). The reliability of the method and protocol is supported by the similarity of the data collected in the fiber during early and later contractions. The protocol appears valid in that when true steady-state levels of tension generation are used the stepping and bracketing protocols yield similar data.

The maximum Ca^{2+}-activated tensions were determined at each $[Mg^{2+}]$ and do not vary in absolute value as $[Mg^{2+}]$ is changed even though more Ca^{2+} is required to achieve maximum tension generation as $[Mg^{2+}]$ is increased. However, the maximum Sr^{2+}-activated tension is only approximately 90% of the corresponding maximum Ca^{2+}-activated tension at $[Mg^{2+}] = 5 \times 10^{-5}$ M and 2×10^{-3} M.

Increasing $[Mg^{2+}]$ from 5×10^{-5} M to 2×10^{-3} M results in a shift of both the pCa and the pSr versus percentage of maximum tension curves obtained at $[Mg^{2+}] = 5 \times 10^{-5}$ M. Figs. 3 and 4 show the pCa and pSr versus tension curves, respectively. The symbols represent means of the raw data and the solid lines are computer fittings of the curves (as discussed in the legends). In these two figures it can be seen that the shift in the curves is in the direction of greater $[Ca^{2+}]$ and $[Sr^{2+}]$ and occurs to the greatest extent as $[Mg^{2+}]$ increases from 5×10^{-5} M to 1×10^{-3} M. The shift of the curves as $[Mg^{2+}]$ increases from 1×10^{-3} to 2×10^{-3} M can also be seen from the information in Tables II and III. In Table II the means of the percentages of maximum Ca^{2+}-activated tensions and the standard errors of the means at each $[Mg^{2+}]$ can be compared in any row, according to pCa. Table III is the corresponding one for Sr^{2+}-activated tensions. It is evident that both the pCa and pSr versus tension curves become less steep as $[Mg^{2+}]$ increases.

The reality of the shift of the Ca^{2+} (or Sr^{2+}) curves in going from 1×10^{-3} M to 2×10^{-3} M $[Mg^{2+}]$ was verified by contracting the same fiber at the same submaximum $[Ca^{2+}]$ (or $[Sr^{2+}]$) at both $[Mg^{2+}]$'s. It was found that the percentage of the maximum tension generated by a fiber at $[Mg^{2+}] = 1 \times 10^{-3}$ M was consistently greater than that generated at $[Mg^{2+}] = 2 \times 10^{-3}$ M.

The Sr^{2+} curve is consistently displaced, relative to the Ca^{2+} curve, in the

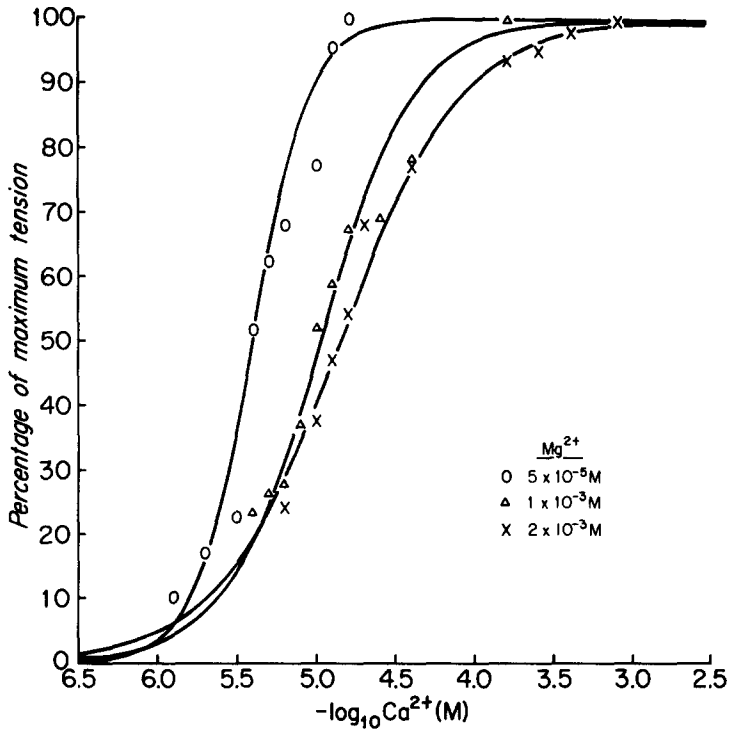

FIGURE 3. Percentages of maximum Ca^{2+}-activated tensions at three Mg^{2+} concentrations. Solid lines are computer fittings of curves to means of raw data (symbols). See Table II for standard errors of the means and sample sizes. The means of raw data (symbols) were fit using the following form of the Hill equation

$$\log_{10}\left(\frac{\%T}{100 - \%T}\right) = n\log_{10}[Ca^{2+}] - \log_{10}Q,$$

where $\%T$ refers to percentage of maximum tension, n and Q are constants. Q and $n \pm$ SD values are displayed, for each set of means, in Table IV. The solid curves are computer fittings derived using these values.

direction of greater concentrations. A comparison of the difference between the curves in $-\log_{10}$ units (p units) at approximately the 50% of maximum tension generation point on each curve shows that the difference is 0.7 p units at $[Mg^{2+}] = 5 \times 10^{-5}$ M and 1×10^{-3} M and 0.6 p units at $[Mg^{2+}] = 2 \times 10^{-3}$ M. Although the Sr^{2+} and Ca^{2+} curves at $[Mg^{2+}] = 5 \times 10^{-5}$ M are almost identical in shape the Sr^{2+} curve appears increasingly steeper *relative* to the Ca^{2+} curve as $[Mg^{2+}]$ increases from 1×10^{-3} M to 2×10^{-3} M.

Qualitatively similar results were obtained in earlier experiments where no CP or CPK were used in that maximum tension was not a function of $[Mg^{2+}]$ and that similar changes in $[Mg^{2+}]$ yielded a statistically significant difference in the $[Ca^{2+}]$ and $[Sr^{2+}]$ required for 50% of maximum isometric tension

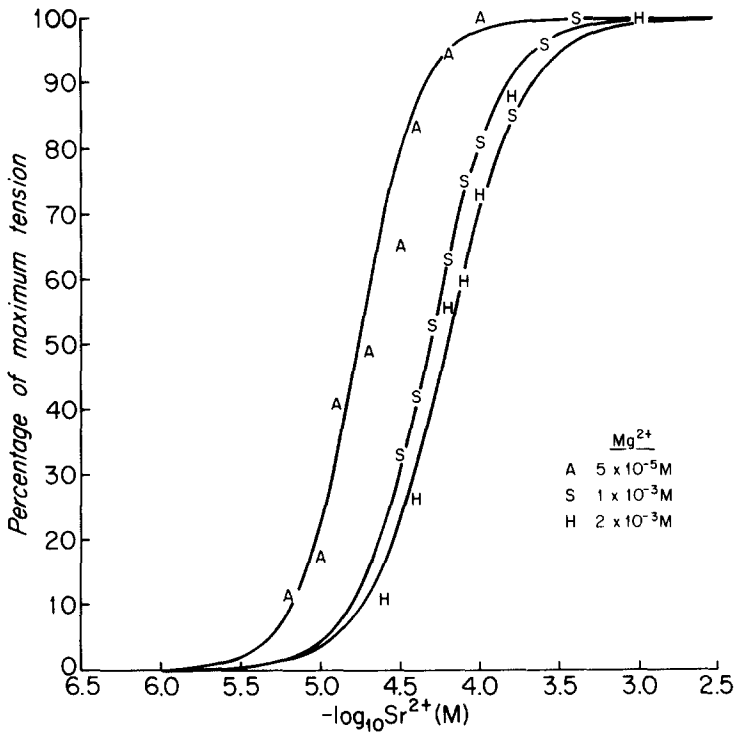

FIGURE 4. Percentages of maximum Sr^{2+}-activated tensions at three Mg^{2+} concentrations. Solid lines are computer fittings of curves to means of raw data (symbols). See Table III for standard errors of the means and sample sizes. The means of raw data (symbols) were fit using the following form of the Hill equation

$$\log_{10}\left(\frac{\%T}{100 - \%T}\right) = n\log_{10}[Sr^{2+}] - \log_{10}Q,$$

where $\%T$ refers to percentage of maximum tension, n and Q are constants. Q and n ± SD values are displayed, for each set of means, in Table IV. The solid curves are computer fittings derived using these values.

generation. Also, data from these earlier experiments were similar in that the relative sensitivity of the tension-generating system to Ca^{2+} versus Sr^{2+} did not change as $[Mg^{2+}]$ increased. The data from these preliminary experiments were not included because of differences discussed later.

DISCUSSION

Effects of Changes in $[Mg^{2+}]$

Mg^{2+} does not affect maximum tension generation but since it does exert a depressant action on Ca^{2+}-activated submaximum tension generation, it is possible that Mg^{2+} has a role in modulating muscle contraction. Fluctuations

TABLE II

MEANS OF PERCENTAGES OF MAXIMUM TENSIONS AND STANDARD ERRORS OF THE MEANS ACCORDING TO pCa AND $[Mg^{2+}]$

	Means of percentages of maximum tensions ± SEM and sample size (*n*) at each $[Mg^{2+}]$		
pCa	$[Mg^{2+}] = 5 \times 10^{-6}$ M	$[Mg^{2+}] = 1 \times 10^{-3}$ M	$[Mg^{2+}] = 2 \times 10^{-3}$ M
5.9	10.3±0.3 (3)		
5.7	17.2±1.1 (6)		
5.5	22.8±2.0 (6)		
5.4	51.8±2.8 (6)	23.5±1.5 (2)	
5.3	62.4±3.4 (5)	26.7±4.0 (4)	
5.2	68.0±3.3 (8)	28.0±2.7 (3)	24.2±4.9 (4)
5.1		37.3±2.4 (3)	
5.0	77.4±2.7 (8)	52.3±1.9 (7)	37.8±3.4 (6)
4.9	95.7±2.3 (6)	59.0±1.7 (9)	47.2±1.3 (12)
4.8	100	67.5±2.5 (4)	54.4±3.8 (11)
4.7			68.2±5.3 (5)
4.6		69.3±1.2 (3)	
4.4		78.7±1.8 (3)	77.2±3.7 (5)
3.8		100	93.7±3.4 (3)
3.6			95.3±2.5 (3)
3.4			98.0±1.4 (2)
3.1			100

TABLE III

MEANS OF PERCENTAGES OF MAXIMUM TENSIONS AND STANDARD ERRORS OF THE MEANS ACCORDING TO pSr AND $[Mg^{2+}]$

	Means of percentages of maximum tensions ± SEM and sample size (*n*) at each $[Mg^{2+}]$		
pSr	$[Mg^{2+}] = 5 \times 10^{-6}$ M	$[Mg^{2+}] = 1 \times 10^{-3}$ M	$[Mg^{+2}] = 2 \times 10^{-3}$ M
5.2	11.7±2.0 (3)		
5.0	17.5±3.7 (4)		
4.9	41.0±4.6 (5)		
4.7	49.0±4.7 (6)		
4.6			11.0±1.5 (3)
4.5	65.3±3.6 (9)	33.3±2.6 (3)	
4.4	83.4±2.0 (7)	42.3±3.9 (3)	26.6±5.5 (5)
4.3		53.1±2.9 (9)	
4.2	94.6±1.2 (7)	63.2±2.8 (11)	55.7±2.2 (15)
4.1		75.0±3.3 (5)	59.8±2.3 (5)
4.0	100	81.0±0.7 (4)	73.0±2.1 (5)
3.8		85.0±1.5 (3)	88.0±6.2 (3)
3.6		96.0±4.0 (2)	
3.4		100	
3.0			100

in [Mg^{2+}] during the contraction-relaxation cycle could enhance or depress Ca^{2+}-activated tension generation independent of changes in the concentration of any other ions such as [ATP^{4-}], [$MgATP^{2-}$], and [$CaATP^{2-}$] (Kerrick and Donaldson, 1972). In addition the changes in shape of the percentage of maximum tension versus pCa curve means that at a given level of submaximum tension generation the effect of a given fractional change in [Ca^{2+}] on tension generation varies inversely with the [Mg^{2+}]. (Maximum tension generation, however, would not be affected by changes in [Mg^{2+}] unless the higher [Ca^{2+}]'s required for maximum tension generation at higher [Mg^{2+}]'s were not achieved.)

The range of [Ca^{2+}] associated with minimum to maximum tension generation at [Mg^{2+}] = 1×10^{-3} M and 2×10^{-3} M in this study seems to be slightly higher than what is usually thought of as "physiological." Winegrad (1970) estimated from autoradiographic measurements that the maximum [Ca^{2+}] which could possibly be achieved in vivo during tetanic contraction is approximately 2×10^{-4} M or pCa = 3.7. Also the [Ca^{2+}] versus tension curves of this study are shifted in the direction of higher [Ca^{2+}]'s in comparison to our earlier data (Kerrick and Donaldson, 1972). This shift is probably not due to the decrease in ionic strength since our earlier data indicated this should make the system more sensitive to Ca^{2+}. As discussed earlier changes in K^+ and imidazole concentration do not affect the Ca^{2+} sensitivity of the system. It is most likely that the CP-CPK regenerating system created this discrepancy, in part by eliminating gradients in [$MgATP^{2-}$] within the fiber during contraction (Godt, 1974). If the [MgATP] were decreased in the interior of the fibers in the earlier study the system would be significantly more sensitive to Ca^{2+} (Brandt et al., 1972; Godt, 1974).

Thus, although the changes in Ca^{2+} sensitivity demonstrated in this study are not the result of variations in [K^+], [$MgATP^{2-}$], ionic strength, etc., their absolute values may affect the absolute position of the saturation curve along the [Ca^{2+}] (or pCa) axis. In view of this no assertion is made that the range of [Ca^{2+}]'s in this study defines physiological range, but rather that the nature of the effects of [Mg^{2+}] on the system are similar to those in vivo.

Nature of Mg^{2+} Inhibition

The tension in the curves of Figs. 3 and 4 rises from 10 to 90% of maximum in less than 2 pCa (or pSr) units which is indicative of interacting sites. Because of the evidence for interacting sites these data were analyzed using the Hill equation (Hill, 1913) which accounts for cooperative forces in the binding of ligand to a macromolecule (Hill, 1913; Loftfield and Eigner, 1969).

A value of Hill n (see legends of Figs. 3 and 4) greater than 1 is indicative of two or more interacting sites per macromolecule, but the value of n is a lower limit for rather than the exact number of binding sites per mole of protein.

A decrease in the value of n down to a lower limit of just greater than 1 is indicative of a change in degree of cooperativity (Wyman, 1963; Koshland et al., 1966). As seen in Table IV, n values are greater than 1 except for Ca^{2+} at $[Mg^{2+}] = 2 \times 10^{-3}$ M where n is not different from 1.

Since the maximum Ca^{2+}- (or Sr^{2+}-)activated tension is not a function of $[Mg^{2+}]$, Mg^{2+} does not appear to be an inhibitor which alters the number of binding sites. However, it does appear to affect the affinity of the activating sites either directly or indirectly. The pCa_{50}-pSr_{50} difference remains the same as $[Mg^{2+}]$ increases which is consistent with competitive inhibiton by Mg^{2+} or indirect effects of Mg^{2+} on the affinity of the activating sites. Indirect effects might be linked to Mg^{2+}'s effects on cooperativity.

Using too large an association constant for MgCP (K_{MgCP}) in calculating the ionic equilibria for the bathing solutions would tend to decrease the

TABLE IV

HILL COEFFICIENTS ± STANDARD DEVIATIONS OF HILL COEFFICIENTS AND CONSTANTS OF PERCENTAGE OF MAXIMUM TENSION CURVES FOR Ca^{2+} AND Sr^{2+} AT EACH $[Mg^{2+}]$

	$[Mg^{2+}]$					
	5×10^{-5} M		1×10^{-3} M		2×10^{-3} M	
Divalent cation	$n \pm$*	Q(M)	$n \pm$*	Q(M)	$n \pm$*	Q(M)
Ca^{2+}	2.47±0.30	$10^{-13.38}$	1.50±0.12	$10^{-7.46}$	1.14±0.036‡	$10^{-5.52}$
Sr^{2+}	2.31±0.19	$10^{-10.96}$	1.97±0.12	$10^{-8.52}$	1.80±0.11	$10^{-7.54}$

* Sample standard deviation of n.

‡ For a 99.5% confidence interval, n is greater than one except for Ca^{2+} at $[Mg^{2+}] = 2 \times 10^{-3}$ M where it is not different from 1. Decreasing n to 1 indicates a decrease in cooperativity (Wyman, 1963; Koshland et al., 1966).

pCa_{50}-pSr_{50} difference as $[Mg^{2+}]$ increased but not enough to result in the incorrect adoption of a model where Mg^{2+} affects the affinity of the activating sites indirectly or by competition. Also, while collecting some preliminary data for this study using similar solutions but with $K^+ = 90$ mM, ionic strength = 0.20 M, and no CP or CPK (see Kerrick and Donaldson, 1972, for solutions' constituents) it was found that the pCa_{50}-pSr_{50} difference remained constant as $[Mg^{2+}]$ was increased from 0.3×10^{-3} M to 2×10^{-3} M. In this case there could be no effect of an error in the K_{MgCP}.

In order to account for the effects on cooperativity and Ca^{2+} affinity of the system, Mg^{2+} must bind to at least one site which is separate from the Ca^{2+} activating sites. There is some evidence for Mg^{2+} competing for Ca^{2+} sites on troponin (Potter and Gergely, 1974) but no evidence of interaction of binding sites on isolated troponin. However, most of these binding studies have been done at high $[Mg^{2+}]$ (Weber and Murray, 1973) and the relationship of tension and Ca^{2+} binding to these sites is not known.

Mg^{2+} might also be affecting Ca^{2+} binding to myosin sites. Bremel and Weber (1975) have noted that increasing [Mg^{2+}] from 3 μM to 5 mM alters the degree of cooperativity and decreases the sensitivity of Ca^{2+} binding sites on rabbit myosin.

The reason for the maximum Sr^{2+}-activated tension being only 90% of the maximum Ca^{2+}-activated tension is not understood. It is possible that it is due to some concentration effect of activating divalent cation (Ca^{2+} or Sr^{2+}) concentration since when [Ca^{2+}] was increased beyond maximum (into the concentration range required for maximum Sr^{2+}-activated tension generation) the steady-state Ca^{2+}-activated tension generation also decreased to approximately 90% of maximum.

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