The Regulation of Tension in a Chemically Skinned Molluscan Smooth Muscle

Effect of Mg\(^{2+}\) on the Ca\(^{2+}\)-activated Tension Generation

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ABSTRACT Chemically skinned anterior byssus retractor muscle (ABRM) preparations were prepared by treatment with the nonionic detergents saponin and Triton X-100. Both maximum peak tension and rate of contraction were found to be greater in saponin-treated ABRM than in ABRM treated with Triton X-100. Active tension was initiated at a concentration of free Ca\(^{2+}\) above 0.1 \(\mu\)M, and maximum tension development was found at a \([Ca^{2+}] \approx 32 \mu\)M. During exposure of the muscle preparation to optimal Ca\(^{2+}\) concentration, a high and almost constant tension level was sustained. The force recovery was high after a quick release during this period indicating the presence of an "active" state rather than a "catch" state. Actually, a state equivalent to the catch state in the living ABRM could not be induced, if the Ca\(^{2+}\) concentration was above 0.1 \(\mu\)M. Variations in the ionic strength in the range of 0.07-0.28 M had no influence on active state and only slightly affected the maximum tension developed. The influence of Mg\(^{2+}\) on the Ca\(^{2+}\)-activated tension was examined by studying the tension-pCa relation at two concentrations of free Mg\(^{2+}\) (0.43 and 4.0 mM). The tension-pCa relation was found to be S-shaped with tension increasing steeply over ~1 pCa unit, indicating the existence of cooperativity between Ca\(^{2+}\) sites. Increasing the free concentration of Mg\(^{2+}\) shifted the tension-pCa relation to lower pCa as in striated muscles, demonstrating a decreasing Ca\(^{2+}\) sensitivity with increasing Mg\(^{2+}\). At [Mg\(^{2+}\)] = 4.0 mM the half-maximum tension was found at [Ca\(^{2+}\)] = 0.43 \(\mu\)M, decreasing to 0.20 \(\mu\)M at [Mg\(^{2+}\)] = 0.43 mM. At both Mg\(^{2+}\) concentrations studied, plots of \(P_{\text{sat}}/(1 - P_{\text{sat}})\) vs. log [Ca\(^{2+}\)] were nonlinear with a shape indicating a rather complicated model for cooperativity, probably involving four sites for Ca\(^{2+}\). These Ca\(^{2+}\)-Mg\(^{2+}\) interactions are most probably taking place at the myosin head itself because troponin is absent in this myosin-regulated muscle.

INTRODUCTION

Treatment of smooth muscle fiber bundle preparations by various detergents has been used to disrupt the membrane and thereby remove the diffusion...
barrier (chemical skinning). The use of such preparations has permitted the study of the effect of various ionic species on the contraction-relaxation cycle in a state close to the living state. However, lack of reproducibility, retardation of contraction and relaxation, and other problems have often been encountered in using chemically skinned smooth muscle fiber preparations.

Although Mg$^{2+}$ is known to be necessary for muscle contraction, its exact role is still debated. In skinned skeletal muscle fibers Mg$^{2+}$ decreases the Ca$^{2+}$ sensitivity of the contractile apparatus; increasing the [Mg$^{2+}$] in the millimolar range causes a shift in the tension-pCa relation in the direction of higher Ca$^{2+}$ concentrations (cf. Donaldson and Kerrick, 1975). Troponin has been shown to bind both Ca$^{2+}$ and Mg$^{2+}$ (Potter and Gergely, 1975), and it is likely that the Mg$^{2+}$ effects are mediated through binding to the troponin-tropomyosin regulating system in striated muscle.

In molluscan smooth muscle, contraction is regulated via the dithionitrobenzene light chains (LC) of the myosin head (myosin-linked regulation), and tropomyosin is not involved in the Ca$^{2+}$ regulation (Kendrick-Jones et al., 1970). Although functional troponin exists in these muscles, it is probably present in insufficient amount to regulate the thin filaments (Lehman and Szent-Györgyi, 1975; Goldberg and Lehman, 1978).

Mg$^{2+}$ is important in molluscan smooth muscle for the preservation of thick filament structure and for the stabilization of interaction between myosin light and heavy chains (Kendrick-Jones et al., 1976). The LCs from scallop bind Ca$^{2+}$ and, possibly, also Mg$^{2+}$; however, the exact number of binding sites on the functional unit and the magnitude of the binding constants are not known (Kendrick-Jones and Jakes, 1977).

The purpose of the present study is to investigate the effect of Mg$^{2+}$ on the regulation of contraction in a myosin-regulated smooth muscle, namely the anterior byssus retractor muscle (ABRM) of *Mytilus edulis*. In order to do this, the dose-response curves (tension-pCa relations) were determined at two Mg$^{2+}$ concentrations (0.4 and 4.0 mM) by using chemically skinned ABRM preparations. The results were analyzed as described by Cornish-Bowden and Koshland (1975). This procedure (see Appendix) makes it possible to obtain information about site interaction in a multisite system by using the general saturation equation, the Adair equation (see Whitehead, 1970), and by diagnostic uses of the so-called Hill plots.

**METHODS**

Small specimens of the blue mussel, *Mytilus edulis*, were collected from local beaches and kept in an aquarium at 4°C. Usually animals with a shell length of ~20 mm were used. ABRMs from such animals were between 7 and 10 mm long and weighed ~2-3 mg.

**Dissection and Mounting**

The ABRMs were isolated as previously described (Cornelius and Lowy, 1978). In order to obtain sufficient thin muscle preparations each muscle was split longitudinally under a stereomicroscope using fine needles. The final muscle preparations, still
attached to a piece of shell at the anterior end, had the form of flat sheets with a maximum thickness of ~100 μm and a width between 100 and 300 μm.

A short gold chain was tied to the free end of the muscle preparation using 0.5 silk, and the piece of shell fixed to the muscle chamber base. The muscle preparation was suspended vertically by connecting the gold chain to a force transducer (DSC-6, Kistler-Morse Corp., Bellevue, Wash.) whose position could be varied by a micrometer arrangement. The compliance of this setup was less than 1 μm/mN, including connections to the muscle.

The muscle chamber contained 4 ml and was perfused at a rate of 5 ml/s with seawater containing 2.5 × 10⁻⁵ M 5-hydroxytryptamine (5-HT) to eliminate artificial resting tension (Lowy and Millman, 1963). The muscle chamber could be emptied and refilled within 1 s. Before connecting the muscle preparation to the force transducer, it was allowed to hang with a 5 g load for 1 h to equilibrate mechanically. Before the start of an experiment the muscle length was set to Lₒ which was defined as the maximum length at which no resting tension was present (Cornelius and Lowy, 1978). Unless otherwise stated, all experiments were performed at room temperature (20–22°C). The temperature in the muscle chamber was recorded throughout the experiment via a thermistor placed near the muscle preparation.

**Solutions**

The force-producing capacity of the muscle preparation was investigated at varying free Ca²⁺ concentrations, a MgATP²⁻ concentration of 3.5 mM, and at two fixed free Mg²⁺ concentrations. Accordingly, the experimental solutions were prepared by varying the total concentrations of calcium, ATP, and magnesium in such a way that the free concentration of Mg²⁺ was either 0.43 or 4.0 mM.

The total concentrations of the different chemicals needed to produce desired free concentrations of Ca²⁺, Mg²⁺, and MgATP²⁻ were calculated on a minicomputer (Prime 300, Prime Computer Inc., Framingham, Mass.) by using an iterative procedure to solve the binding equations that use the stability constants given in Table I.

The following standard solutions were used: wash solution (W), relaxing solutions (R), and contracting solutions (C). All solutions contained potassium methanesulfonate, magnesium methanesulfonate, Na₂ATP, and 20 mM piperazine-N,N'-bis-(2-ethanesulfonic acid) (PIPES, pK = 6.8 at 20°C) as a pH buffer. W and C solutions

| Table I | VALUES FOR THE ASSOCIATION CONSTANT (Kₐ) USED IN THE COMPUTATIONS |
|---------|--------------------------------------------------------------------|
| Reaction | log Kₐ | Reaction | log Kₐ |
| H⁺ + EGTA³⁺ ≡ HEGTA²⁻ | 9.43 | H⁺ + ATP³⁻ ≡ HATP²⁻ | 7.02 |
| H⁺ + HEGTA³⁻ ≡ H₂EGTA²⁻ | 8.85 | H⁺ + HATP³⁻ ≡ H₂ATP²⁻ | 4.02 |
| H⁺ + H₂EGTA²⁻ ≡ H₂EGTA⁻ | 2.68 | | |
| H⁺ + H₂EGTA⁻ ≡ H₂EGTA⁻ | 2.00 | | |
| Ca²⁺ + EGTA³⁻ ≡ CaEGTA²⁻ | 11.00 | Ca²⁺ + ATP³⁻ ≡ CaATP²⁻ | 4.32 |
| Ca²⁺ + HEGTA³⁻ ≡ CaHEGTA⁻ | 5.33 | Ca²⁺ + HATP³⁻ ≡ CaHATP²⁻ | 2.13 |
| Mg²⁺ + EGTA³⁻ ≡ MgEGTA²⁻ | 5.21 | Mg²⁺ + ATP³⁻ ≡ MgATP²⁻ | 4.65 |
| Mg²⁺ + HEGTA³⁻ ≡ MgHEGTA⁻ | 3.37 | Mg²⁺ + HATP³⁻ ≡ MgHATP²⁻ | 2.65 |

* Bjerrum et al., 1957.  
† Taqui Khan and Martell, 1966.
contained 2 mM ethylene glycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA), and R solution contained 20 or 50 mM EGTA. The pH was adjusted to 6.8 at 20°C. The pCa (−log_{10}[Ca^{2+}]) of the contracting solutions was varied between 8.65 and 3.0.

The ionic strength (I) of the solutions was kept constant at 0.14 M at the expense of constant osmolarity by varying the potassium methanesulfonate concentration. In some experiments the ionic strength was adjusted to either 0.07 or 0.28 M instead of 0.14 M. For the purpose of these experiments (see Results) correction of the stability constants was considered unnecessary.

In some experiments an ATP-regenerating system composed of creatine phosphate (CP, 15 mM) and creatine phosphokinase (CPK, 50 IU/ml) was added to the solutions.

The Chemical Skinning Procedure

The membranes of ARBM preparations were disrupted (chemically skinned) by treatment with the nonionic detergents Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) or saponin (Sigma Chemical Co., St. Louis, Mo.). The procedure for skinning with Triton X-100 followed that of Baguet and Marchand-Dumont (1975). The procedure for disrupting the membrane using saponin was as follows: the skinning solutions were prepared by adding 0.05% (wt/vol) saponin to the wash solution ($S_2$) and to the relaxing solution ($S_{20}$). After an initial tonic contraction of the living muscle preparation using 5.5 × 10^{-5} M ACh in seawater (maximum tension reference), the preparation was washed in seawater containing 5-HT. The completely relaxed ARBM was then exposed to saponin for 15 min. Firstly $S_2$ was applied for 5 min; the preparation responded with a K^+ contracture followed by a spontaneous partial relaxation. Then to obtain complete relaxation the $S_2$ solution was changed to $S_{20}$ solution; a fast and almost complete relaxation was now obtained (see Fig. 1). After 15 min in skinning solutions, the preparation was washed thoroughly with wash solution and relaxing solution.

General Experimental Procedure and Data Analysis

After chemically skinning the muscle preparation it was exposed to relaxing solution. The tension level reached in this solution was used as zero tension reference. To obtain dose-response curves (tension-pCa relations), the muscle preparation was exposed to contracting solutions of increasing free calcium concentrations, thus allowing the tension to reach steady-state level at each Ca^{2+} concentration. This experimental protocol gave the same results as experiments in which the preparation was allowed to relax before the application of each new contracting solution with increasing Ca^{2+}. The steady-state tension level at each pCa was expressed relative to the maximum tension the preparation developed in the experiment. In the analysis of the data a diagnostic use of the so-called Hill plots was employed as suggested by Cornish-Bowden and Koshland (1975). These are log-log plots of $P_{ml}/(1-P_{ml})$ vs. the free calcium concentration, [Ca^{2+}] (see Appendix).

RESULTS

Fig. 2 shows that, for muscle preparations with a maximum diameter of 100–300 μm, exposure to skinning solution for ~8–20 min was optimal. In using such preparations the maximum tension developed in contracting solution compared to the initial (tonic) ACh response of the living preparation was 0.70 ± 0.13 (mean ± SEM, n = 32). The maximum peak tension developed by
skinned preparations, $P_{\text{max}}$, ranged from 600 to 900 mN/mm$^2$. The maximum rate of contraction was found to be $2 \times 10^{-2}$ to $4 \times 10^{-2}$ $P_{\text{max}}$/s, when the EGTA concentration was equal in the wash and in the contracting solution. By improving the buffer conditions (see Ashley and Moisescu, 1975), it was possible to increase the rate of tension development. With an EGTA concentration of 0.2 mM in the wash solution and 20 mM in the contracting solution, the rate of contraction was increased to $\sim 0.1$ $P_{\text{max}}$/s compared to the rate of tension development in an ACh response of the living ABRM, which is $\sim 0.5$ $P_{\text{max}}$/s. The rate of tension development was also measured as the maximum rate of tension recovery after a quick release in order to eliminate rate-limiting effects from diffusion of Ca$^{2+}$ or MgATP$^{2-}$. In detergent-treated preparations this figure was 0.3 $P_{\text{max}}$/s compared to the rate of redeveloped tension after a quick release in an ACh response of a living ABRM of $\sim 1$ $P_{\text{max}}$/s.
Using the skinning procedure of Baguet and Marchand-Dumont (1975) with the detergent Triton X-100 (0.1% wt/vol), both the maximum peak tension developed and the maximum rate of contraction were much lower than in experiments using saponin. Compared to the initial ACh response of the living ABRM, the relative tension developed here was 0.49 ± 0.04 (n = 12) and the maximum rate of contraction was $(6.1 \times 10^{-3} \pm 0.6 \times 10^{-3}) \frac{P_{\text{max}}}{s}$ (n = 15), when measured with 2 mM EGTA in the wash and contracting solutions. In order to investigate whether or not ATP depletion could be a problem under the experimental conditions used, an ATP regenerating system, CP-CPK, was added to the standard solutions in control experiments. However, no changes in the contraction-relaxation cycle could be detected; the maximum peak tension that was developed and the maximum rate of tension development were the same as without the ATP-regenerating system. But it was noted that with the ATP-regenerating system present the reproducibility of the tension responses was improved. Therefore, in prolonged experiments CP-CPK was always present.

**Calcium-Induced Tension Generation and Active State**

When the detergent-treated ABRM preparations were exposed to high $\text{Ca}^{2+}$ concentration, the tension increased up to a plateau and was maintained.

![Figure 2](image_url)
during the presence of high Ca$^{2+}$. During the tension maintenance the muscle preparation was in the active state; after a quick release the preparation redeveloped a large fraction of the active tension held before the release. As seen from Fig. 3, both active tension and active state were maintained almost constant for hours when the muscle preparation was exposed to contracting solution. Removing ATP from the contracting solution caused a relatively small decrease in tension whereas active state almost disappeared (Fig. 4). Addition of ATP again restored the tension as well as the active state. Similar results were obtained using Triton X-100-treated preparations.

![Figure 3](https://example.com/figure3)

**Figure 3.** The time-course of maximum tension production and active state during exposure of the skinned ARBM preparation to contracting solution (pCa = 4.5, I = 0.14 M, pH = 6.8). Inset shows schematically how the parameters were measured. $P_{\text{max}}$ is the maximum tension developed. During the tension plateau a hand-made quick release (QR) of $0.03 L_0$ was applied and the redeveloped tension was measured ($P_2$). $P_1$ level—a measure of instantaneous elasticity—is also indicated.

**Ionic Strength**

The ionic strength has previously been demonstrated to play an important role in the contraction-relaxation cycle for ABRM preparations treated with Triton X-100 or EDTA (Baquet and Marchand-Dumont, 1975). In the present study the effect of ionic strength on tension development and active state was investigated at three different ionic strengths; $I = 0.07, 0.14,$ and $0.28$ M, respectively.

The ABRM preparations were chemically skinned by using skinning solutions adjusted to the appropriate ionic strength. After the skinning the characteristics of the contractions were determined by successive application of contracting solution at all three ionic strengths. As seen from Table II, it
was found that at lower ionic strength the maximum tension development tended to be greater than at the high ionic strength.

At all ionic strengths the ABRM preparation was able to maintain a high tension level for hours in the presence of high Ca\(^{2+}\), and tension was always maintained in the presence of active state (judged from quick-release experiments). A state equivalent to the catch state (Jewell, 1959) could not be initiated when Ca\(^{2+}\) was present in concentrations higher than 10\(^{-7}\) M. The same results were obtained if contracting solutions (pCa = 6) identical to those used by Baguet and Marchand-Dumont (1975) to initiate the catch state in Triton X-100 or EDTA-treated ABRM were tested; i.e., I = 0.07 M, pH = 6.5, and I = 0.28 M, pH = 7.0.

**Effect of Varying Mg\(^{2+}\) at Fixed MgATP\(^{2-}\) on the Tension-pCa Relation**

Mg\(^{2+}\) is known to be necessary for contraction; however, Ca\(^{2+}\) is the primary activator for actomyosin interaction (Ebashi and Endo, 1968). The role of Mg\(^{2+}\) in contraction is still debated.

Fig. 5 shows the relation between relative isometric steady-state tension and pCa at two different free-Mg\(^{2+}\) concentrations: 0.43 mM (△) and 4.0 mM (□). In both cases [MgATP\(^{2-}\)] = 3.5 mM, pH = 6.8, and I = 0.14 M. At both Mg\(^{2+}\) concentrations the symbols represent means of data for six ABRM preparations. Each muscle served as its own control; i.e., the submaximum tensions were expressed as fractions of the maximum tension the muscle preparation could develop. The muscle preparations showed little sign of fatigue judged from the tension responses which were reduced by < 5% within
TABLE II

THE MAXIMUM TENSION DEVELOPED BY CHEMICALLY SKINNED ABRM EXPOSED TO CONTRACTING SOLUTION (pCA = 4.5) OF DIFFERENT IONIC STRENGTH (I)

| Skinning solution, I [M] | Contracting solution, I [M] |
|-------------------------|-----------------------------|
| I [M]                   | 0.07                        | 0.14                        | 0.28                        |
|                         | 0.80±0.09                   | 0.73±0.03                   | 0.64±0.07                   |
| (n=4)                   | (n=16)                      | (n=6)                       |
| 0.14:                   | 0.63±0.03                   | 0.65±0.04                   | 0.55±0.04                   |
| (n=13)                  | (n=13)                      | (n=19)                      |
| 0.28:                   | 0.55±0.06                   | 0.60±0.04                   | 0.54±0.07                   |
| (n=9)                   | (n=5)                       | (n=10)                      |

The ABRM used were chemically skinned at one of the three ionic strengths indicated. Tensions are expressed as fractions of the maximum peak tension developed immediately before skinning stimulating with 5.5 × 10⁻⁵ M ACh. Data are given as mean ± SEM (n = number of observations).

Figure 5. Relationship between relative tension and concentration of free Ca²⁺ (given as pCa). Means of data obtained at two concentrations of Mg²⁺ are shown (□, 4.0 mM; △, 0.43 mM). The lines are drawn by eye through means of experimental data. At each Mg²⁺ concentration six muscle preparations were used. Vertical bars represent mean ± SEM (n = 6).
the course of an experiment. Furthermore, the shape of the tension-pCa relation was unaffected if the experiment was repeated using the same muscle preparation. As seen in Fig. 5 half-maximum tension was generated at $2 \times 10^{-7}$ M Ca$^{2+}$ at $[\text{Mg}^{2+}] = 0.43$ mM. The Ca$^{2+}$ concentration needed to produce half-maximum tension was increased to $4.3 \times 10^{-7}$ M when the free concentration of Mg$^{2+}$ was raised to 4.0 mM.

In Fig. 6 are shown Hill plots of experimental data obtained at the two concentrations of free Mg$^{2+}$. In both cases the relationship of $\log \left( \frac{P_{\text{rel}}}{(1-P_{\text{rel}})} \right)$ vs. $\log [\text{Ca}^{2+}]$ is nonlinear.

Deactivation and the Effect of Mg$^{2+}$ at pCa $\leq 4$

From Fig. 5 it is seen that maximum tension was generated at pCa $\approx 4$ at both Mg$^{2+}$ concentrations used. Increasing the Ca$^{2+}$ concentration further (pCa $\leq 4$) leads to a very pronounced (steady state) deactivation. When an ABRM preparation equilibrated at a high Ca$^{2+}$ concentration was exposed to contracting solution with a supraoptimal Ca$^{2+}$ concentration (pCa $\leq 4$), the tension immediately decreased to a new and lower steady-state level (see Fig. 1). If, however, the preparation was equilibrated at a low Ca$^{2+}$ concentration (e.g., wash solution) and exposed to a supraoptimal Ca$^{2+}$ concentration, a transient tension development was seen: the tension at first increased, but soon decreased to the same steady-state level as in the experiment mentioned above.

Mg$^{2+}$ was found to influence this deactivation. In Fig. 7 the effect of varying the Mg$^{2+}$ concentration in contracting solutions with supraoptimal Ca$^{2+}$ concentration (pCa $\approx 4$) is seen. High Mg$^{2+}$ concentrations were able to overcome the deactivating effect at pCa $= 4$. Only when the free Mg$^{2+}$ concentration was below 0.4 mM, deactivation at pCa $= 4$ could be observed. At lower Ca$^{2+}$ concentrations than corresponding to pCa $= 4$, no effect of Mg$^{2+}$ could be demonstrated; below $[\text{Ca}^{2+}] = 4$ mM, deactivation could not be initiated by lowering the free Mg$^{2+}$ concentration.

Discussion

In the present study effective skinning of ABRM preparations was obtained using the nonionic detergent saponin. In saponin-treated ABRM the maximum force-producing capacity, $P_0$, ranged from 600 to 900 mN/mm$^2$ and the maximum rate of tension development was about 0.1 $P_0$/s. Both figures are somewhat lower than in untreated living muscle preparations stimulated with ACh (tonic contraction), although they were both much higher than in ABRM treated with the detergent Triton X-100.

In a recent study Tanaka and Tanaka (1979) used saponin-treated ABRM fiber bundles pretreated with glycerol for 2 h and obtained only 160 mN/mm$^2$ for the maximum tension and $\sim 0.05 P_0$/s for the maximum rate of tension rise (estimated from Fig. 1 in Tanaka and Tanaka, 1979). With taenia coli from rabbit Gordon (1978) obtained a tension response from a chemically skinned preparation which was equal in magnitude to the response obtained using electrical stimulation. However, the maximum rate of tension rise was at least 30 times lower in the skinned preparation than in the living preparation (Fig. 1 in Gordon, 1978).
Figure 6. Hill plots of experimental and model data. The lines are best fit obtained from model calculations using the Adair equation (See Appendix) for a multisite protein with two, three, and four sites ($n = 2, 3, \text{and } 4$). Asymptotes of unit slope (---) were located by eye. Panel a compares Hill plots of models and experimental data obtained at $[\text{Mg}^{2+}] = 0.43 \text{ mM (\Delta)}$. The intercepts of the asymptotes on the abscissa axis correspond to: $K_a = 2 \times 10^8 \text{ M}^{-1}$, and $K_a' = 2.5 \times 10^8 \text{ M}^{-1}$. The following binding constants were used in the computation: $n = 2$: $K_1 = 2 \times 10^8 \text{ M}^{-1}; K_2 = 2.5 \times 10^8 \text{ M}^{-1}; n = 3$: $K_1 = 2 \times 10^8 \text{ M}^{-1}; K_2 = 1 \times 10^8 \text{ M}^{-1}; K_3 = 2.5 \times 10^8 \text{ M}^{-1}; n = 4$: $K_1 = 2 \times 10^8 \text{ M}^{-1}; K_2 = 1 \times 10^8 \text{ M}^{-1}; K_3 = 3.5 \times 10^8 \text{ M}^{-1};$ and $K_4 = 2.5 \times 10^8 \text{ M}^{-1}$. Panel b compares Hill plots of models and experimental data obtained at $[\text{Mg}^{2+}] = 4.0 \text{ mM (\square)}$. The intercepts of the asymptotes on the abscissa axis correspond to: $K_1 = 6.3 \times 10^5 \text{ M}^{-1}$ and $K_a' = 2.5 \times 10^6 \text{ M}^{-1}$. Except for $K_a'$, all other constants in the computations of the models were the same as in panel a.
In the present study the differences demonstrated in the behavior of living and chemically skinned muscles could be explained neither by delayed diffusion of Ca\(^{2+}\), because the rate of redeveloped tension after a quick release from a steady-state tension level is also lower in skinned preparations, nor to depletion of MgATP in the center of the preparations caused by the use of too thick fiber bundles; addition of an ATP-regenerating system, CP-CPK, had no effect on either the maximum tension production or the rate of contraction. Theoretical considerations confirm these results. Thus, the external concentration of MgATP (\(C_{ex}\)) needed to give full saturation of a cylindrical muscle cross-sectional area during maximum contraction is given by:

\[
C_{ex} = \frac{K_r^2}{4D},
\]

where \(K = \text{ATPase activity} \), \(D = \text{diffusion constant for MgATP (ATP) in the muscle} \), and \(r_0 = \text{the maximum radius of the muscle cylinder} \). With 3.5 mM MgATP in the external medium, \(K = 80 \text{ nmol} \cdot \text{s}^{-1} \cdot \text{cm}^{-2} \) (20°C, Rüegg, 1971), and \(D = 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1} \) (20°C, Kushmerick and Podolsky, 1969); this gives a limiting radius of \(r_0 = 132 \mu\text{m} \).

The tension response of the detergent-treated preparations was extremely reproducible. Normally, the maximum tension developed by the preparation at the end of an experiment was reduced by at most 5%. In contrast to the results of Endo et al. (1977), who used a saponin-treated taenia caecum, no deterioration in the Ca\(^{2+}\) sensitivity of the skinned ABRM could be detected.
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in experiments where the tension-pCa relations were determined repeatedly in the same preparation.

Chemical skinnning of the ABRM preparation at I = 0.07, 0.14, or 0.28 M, respectively, followed by the application of contracting solution with the same three ionic strengths resulted in slightly higher tensions ($P_{\text{max}}$) the lower the ionic strength (Table II). This tendency is most probably explained by an effect of ionic strength on the contractile protein interaction (Gordon et al., 1973) and not on the effectiveness of chemically skinning, as nonionic detergents are relatively insensitive to salt concentration (Helenius et al., 1979).

At all ionic strengths and pH's studied the application of contracting solution was followed by a parallel development of tension and active state (see Fig. 3). A state equivalent to the catch state in the living ABRM cannot be produced when Ca$^{2+}$ is present in concentrations above 10$^{-7}$ M irrespective of ionic strength and pH used. Thus, the results of Baguet and Marchand-Dumont (1975), who were able to initiate a state where the ABRM produced a high tension without active state in EDTA-treated ABRM, could not be reproduced using neither saponin-treated nor Triton X-100-treated ABRM preparations. A reason for this discrepancy could conceivably be that EDTA is ineffective in rendering the membrane highly permeable to Ca$^{2+}$, as recently demonstrated by Miller (1979). Thus, in the study of Baguet and Marchand-Dumont (1975), the catch state could in reality be a rigor state caused by ATP depletion. In Fig. 4 it is seen that the state of ATP depletion is very similar to the catch state in living ABRM. As suggested by Miller (1979) the high Ca$^{2+}$ sensitivity of EDTA-treated muscle preparations could be the result of a stimulation of a Na$^{+}$-Ca$^{2+}$ exchange system. A similar situation might explain the effect of Na$^{+}$ on the rate of relaxation demonstrated in EDTA-treated ABRM preparations (Baguet, 1977). In fact, a state equivalent to the catch state in the living ABRM could only be induced in saponin-treated ABRM preparations if the Ca$^{2+}$ concentration was below 10$^{-7}$ M. 1

In the tension-pCa relation, tension was found to increase steeply over $\sim$1 pCa unit. The Ca$^{2+}$ concentration needed to produce half-maximum tension ($K_{0.5}$) varied between 2 × 10$^{-7}$ and 4 × 10$^{-7}$ M depending on the Mg$^{2+}$ concentration. These figures are very similar to those found for most striated muscles (Hellam and Podolsky, 1969; Julian, 1971), as well as for vertebrate smooth muscle (Endo et al., 1977; Saida and Nonomura, 1978). In all experiments Ca$^{2+}$ concentrations higher than 10$^{-4}$ M caused a pronounced depression of the steady-state tension (Fig. 1). Similar deactivating effects at high Ca$^{2+}$ concentrations have previously been observed in myofibrils of striated muscles (Portzehl et al., 1969) and in actomyosins of vertebrate smooth muscles (Sobieszek and Small, 1976). Portzehl et al. (1969) explained their results as a primary effect of Ca$^{2+}$ upon the activation, whereas Sobieszek and Small (1976) explained their observations as a result of increased competition between CaATP and MgATP for a substrate site on the myosin ATPase. The deactivation observed in this study occurs at both Mg$^{2+}$ concentrations studied, i.e., in situations where the MgATP/CaATP concentration

1 Cornelius, F. Manuscript in preparation.
ratio is very different. At pCa = 4 the ratio (MgATP/CaATP) is 9 for [Mg\(^{2+}\)] = 0.43 mM, but 900 for [Mg\(^{2+}\)] = 4.0 mM; nevertheless, the same degree of deactivation was found in the two cases (see Fig. 5). This result speaks in favor of deactivation being a direct effect of Ca\(^{2+}\) on the contractile apparatus in ABRM. The observed deactivation is, however, unlikely to play any physiological role since the Ca\(^{2+}\) concentration in the myofibrillar space probably never exceeds 10\(^{-4}\) M. In accordance with this conclusion the deactivation was found to cause irreversible damage to the muscle preparation (Fig. 7).

In skeletal muscle troponin binds Ca\(^{2+}\) and Mg\(^{2+}\) (Potter and Gergely, 1975) and thereby triggers the contraction. In ABRM and other molluscan smooth muscles troponin is found in concentrations insufficient to have a similar function (Lehman and Szent-Györgyi, 1975; Goldberg and Lehman, 1978). In these muscles Ca\(^{2+}\) and Mg\(^{2+}\) probably exhibit their effect by binding to the myosin head itself.

The very steep tension-pCa relation found at both Mg\(^{2+}\) concentrations (Fig. 5) suggest the existence of interactive sites on the functional unit. As seen in Fig. 6 the Hill plots do not give linear relationship between log (Prel/(1-Prel)) and pCa. The Hill equation (see Appendix) can therefore not account for the experimental observations. A more general mathematical model than the Hill equation for binding of Ca\(^{2+}\) to a multisite protein is the Adair equation which accounts for the nonlinearity of the Hill plot (eq. 1 in Appendix). In Fig. 6 a and b the best fit based on model calculations according to the Adair equation for the case of two, three, and four sites on the functional unit have been plotted and compared to the experimental data obtained at the two concentrations of free Mg\(^{2+}\). In order to obtain a reasonable good fit a cooperative model with at least four Ca\(^{2+}\) sites on the functional unit has to be considered. The cooperativity is apparently restricted to the first binding step since K\(_1\), K\(_2\) and K\(_3\) are approximately equal (see Appendix).

The effect of Mg\(^{2+}\) on the Ca\(^{2+}\) sensitivity of the preparation can be satisfactorily described by simple competition between Ca\(^{2+}\) and Mg\(^{2+}\) at one step in the four-site model.

In skeletal muscle the regulation of contraction is attributed to changes in the affinity of the troponin complex for Ca\(^{2+}\), whereas in molluscs the Ca\(^{2+}\) on/off switch is regulated by binding of Ca\(^{2+}\) to the light chains of myosin (Kendrick-Jones et al., 1970). However, myosin light chains from skeletal muscle also bind Ca\(^{2+}\) with two distinct affinities in the absence of Mg\(^{2+}\) in the same range as found here for the ABRM, i.e., with binding constants of 10\(^8\) M\(^{-1}\) and 10\(^6\) M\(^{-1}\) (Watterson et al., 1979). Furthermore, it is interesting to note that in both cases the Ca\(^{2+}\) sensitivity decreases when the Ca\(^{2+}\) concentration increases.

**APPENDIX**

In the analysis of tension-pCa relations it has been common to express the ligand-binding properties of the force producing unit by the Hill equation (Hill, 1910):

\[ P_{rel} = \frac{K[Ca^{2+}]^{nH}}{K[Ca^{2+}]^{nH} + 1}, \]

which only contains two constants K and nH (cf. Donaldson and Kerrick, 1975; Gordon, 1978). The maximum tension production, \(P_{max}\), is then taken to be equivalent
to full saturation of the ligand-binding protein with Ca\(^{2+}\), and fractional saturation (Y) as proportional to the relative tension, \(P_{\text{rel}}\). The Hill equation transforms into a straight line relationship as follows:

\[
\log \frac{P_{\text{rel}}}{1 - P_{\text{rel}}} = \log K + nH \log [\text{Ca}^{2+}],
\]

and the plot of \(\log (P_{\text{rel}}/(1 - P_{\text{rel}}))\) vs. \(\log ([\text{Ca}^{2+}]\) is known as a Hill plot.

It is now known, however, that the theoretical basis for the Hill equation is an oversimplification: in the reaction of ligand (X) with functional unit (E) only E and EX are assumed to exist, whereas all intermediate forms, EX, EX\(_2\), and EX\(_{n-1}\) are negligible. \(n\) is the number of binding sites.

A more general saturation equation for binding of a ligand to a multisite protein is expressed by the Adair equation (Adair, 1925; quoted in Whitehead, 1979):

\[
l = \sum_{i=0}^{n} (EX)_i
\]

\[
y = \frac{\sum_{i=1}^{n} i(EX)_i}{\sum_{i=0}^{n} (EX)_i}
\]

The equation can be rearranged to yield the Hill plot and gives for the case of two, three, and four binding sites (\(n\)) the following equations:

\[
\log \left( \frac{Y}{(1 - Y)} \right) = \log \frac{K_iX + K_iK_2X^2}{1 + K_iX};
\]

\[
\log \left( \frac{Y}{(1 - Y)} \right) = \log \frac{K_iX + 2K_iK_2X^2 + K_iK_2K_3X^3}{1 + 2K_iX + K_iK_2K_3X^3};
\]

\[
\log \left( \frac{Y}{(1 - Y)} \right) = \log \frac{K_iX + 3K_iK_2X^2 + 3K_iK_2K_3X^3 + K_iK_2K_3K_4X^4}{1 + 3K_iX + 3K_iK_2X^2 + K_iK_2K_3X^3};
\]

where \(K_i\) are the intrinsic association constant (site constant) for the \(i\)'th ligand binding step. \(K'_i\) is related to the thermodynamic binding constants \(K_i\) via a static factor:

\[
K'_i = \frac{(n + 1 - i)}{i} K_i.
\]

In contrast to the Hill equation, Eq. 2, 3, 4 do not give a linear relationship between \(\log Y/(1 - Y)\) and \(\log X\); however, at very low and at very high concentrations of ligand, the curves approach two asymptotes of unit slope whose intercept on the abscissa axis has the values of \(-\log K'_i\) and \(-\log K'_i\). Thus, as pointed out by Cornish-Bowden and Koshland (1975), Hill plots can yield a substantial amount of information about the \(K'_i\) values and can be used to distinguish between models for cooperative effects.

In Fig. 6 data for typical experiments at the two Mg\(^{2+}\) concentrations used are plotted in the form of a Hill plot and compared to model calculations according to a multisite protein with two sites (\(n = 2\)), three sites (\(n = 3\)), and four sites (\(n = 4\)) defined by Eqs. 2, 3, and 4, respectively.

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