Calcium Binding by Particle-Free Supernatants of Homogenates of Skeletal Muscle

F. NORMAN BRIGGS and MARTIN FLEISHMAN

From the Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh

ABSTRACT A high molecular weight fraction of a soluble Marsh muscle-relaxing preparation has been shown to contain a calcium-complexing substance. By examining the nature of the competition between this fraction and chelex-100 for calcium at various total calcium concentrations it has been possible to calculate the concentration and calcium stability constant of this calcium-complexing substance. Taking into account dilutions which occur during the preparation of fractions containing this substance its concentration may be estimated at about $2 \cdot 10^{-4}$ in muscle and its calcium stability constant was found to be about $1.5 \cdot 10^{5}$ M^{-1}. Preliminary evidence suggests that the calcium-binding substance is a protein.

The Marsh skeletal muscle-relaxing factor indirectly produces relaxation[1] by the withdrawal of calcium from the contractile mechanism (1–3). It is, therefore, of considerable interest and importance to know which constituents of Marsh factor preparations influence calcium concentration and the mechanisms by which they accomplish this effect. Many of the early experiments (4–7) attempting to identify the material from skeletal muscle involved in producing relaxation indicated that the relaxation activity resided exclusively with the microsome fraction. This is true, however, only when ATP is the energy source for calcium uptake. Weber *et al.* (8) have shown that muscle mitochondria, in the presence of succinate, can also decrease calcium to the level of 10^{-7} to 10^{-6} M Ca^{2+}, the range of calcium concentrations which are limiting for contraction.

The claim that there are soluble substances in skeletal muscle homogenates which can influence actomyosin activity (9–11) in a calcium reversible manner appears controversial. Doubts about the validity of this contention have arisen because the earlier evidence (12–14) that skeletal muscle microsomes

[1] The term relaxation is used to denote a cessation, which is calcium reversible, of such contractile-like phenomena as ATP induced or dependent syneresis, ATPase activity or tension generation in actomyosin containing preparations. Relaxation factors thus differ from the numerous list of substances which can modify the activity of actomyosin preparations by being inactivated by calcium.

produce a soluble inhibitor of actomyosin activity has been shown by Seidel and Gergely (15) to be fallacious. This fallacy arose, in part, because the actomyosin system is sensitive to calcium lack independent of the mechanism of its genesis. In the report that follows a resin system has been developed to test whether the soluble fractions of skeletal muscle homogenates which relax actomyosin preparations are simply calcium deficient or whether they contain, in addition, substances capable of binding calcium. By the use of this resin system evidence has been obtained for such a calcium-binding substance. Furthermore, it has been possible to show that the calcium stability constant of the calcium complex formed with this substance is sufficiently large to effect the sequestration of significant amounts of calcium and to bring ionic calcium concentrations into the range required for the control of contraction.

EXPERIMENTAL PROCEDURES

Preparation of Muscle Fractions

Rabbit hind leg muscle, 230 gm, was chilled in ice and homogenized for 1 minute in 700 ml of solution II (see below). This and all subsequent preparative procedures were carried out in a cold room at approximately 5°C. Myofibrils, debris, and mitochondria were removed by centrifugation at 8000 *g* for 20 minutes. The supernatant from this fractionation was filtered through cheesecloth and centrifuged at 35,000 *g* for 70 minutes. The material in this pellet, constituting the heavy microsome fraction, was suspended in a volume of solution II equal to one-tenth of the volume of the 35,000 *g* supernatant. The 35,000 *g* supernatant was centrifuged at 198,000 *g* for 60 minutes. The supernatant was carefully removed with a Pasteur pipet and the pellet discarded. The 198,000 *g* supernatant served as starting material for three preparations, P-1, P-2, and P-3.

P-1: 100 ml of 198,000 *g* supernatant, 2.5 ml of heavy microsomes and 0.4 mmoles of ATP were incubated together at room temperature for 10 minutes and then subjected to centrifugation, 198,000 *g*, for 60 minutes. This step was introduced to remove traces of calcium from the muscle fraction. The supernatant from this centrifugation was then fractionated on a sephadex G-25 column (50 × 350 mm) equilibrated with solution I. The non-retarded fraction constitutes preparation P-1.

P-2: This preparation was made in a manner identical to that for P-1 except that the sephadex G-25 column was equilibrated with solution II.

P-3: To make this preparation the sequence of steps was reversed so that 100 ml of 198,000 *g* supernatant was first run on a sephadex G-25 column equilibrated with solution II. The non-retarded fraction from the sephadex column was brought to room temperature, heavy microsomes and ATP were added, the mixture incubated for 10 minutes, and then centrifuged at 198,000 *g* for 60 minutes. The supernatant from this centrifugation constitutes preparation P-3.

Calcium-45 Binding

The presence of soluble calcium-binding substances was detected by their sequestration of Ca^{45} which would otherwise have been bound to chelex-100. The method is similar to that used by Schubert (16) to determine stability constants except that the metal concentration has been varied rather than the chelator concentration. This has allowed the determination of both the concentration and stability constant of the calcium-binding substance. The chelex-100 was washed thoroughly in 1 M HCl, brought to pH 6.7 with KOH, equilibrated with 1 M $MgCl_2$, washed extensively with distilled water, and equilibrated by extensive washing with solution I or II depending upon the experiment. The chelex-100 was equilibrated with solution II when used to test the calcium-binding activity of P-3 or P-2 and with solution I when used to test P-1. The chelex-100, 0.5 or 1 ml, was pipeted from a constantly stirred reservoir into 12 ml conical centrifuge tubes. The resin was allowed to settle and the solution in which it was suspended was removed with a Pasteur pipet. The sedimented resin volume, which is indicated for each experiment, was either 0.13 or 0.26 ml. Five ml of P-1, P-2, or P-3 containing 0.3 μc of Ca^{45} and varying amounts of Ca^{40} were added to the centrifuge tubes containing chelex-100 and vigorously stirred for 15 seconds. The resin was then sedimented in a clinical centrifuge. A 0.5 ml sample of the supernatant was removed and its content of radioactivity measured by liquid scintillation using a Packard liquid scintillation spectrometer.

Solutions

The following solutions are defined:

Solution I; 50 mM KCl, 30 mM imidazole 4 mM $MgCl_2$, pH 6.7,
Solution II; solution I with 1 mM potassium oxalate, pH 6.7,
Solution III; solution II with 4 mM ATP, pH 6.7.

Reagents

Sephadex G-25 was obtained from Pharmacia, Upsala, Sweden. The disodium salt of ATP was obtained from General Biochemicals Incorporated. Chelex-100 resin was purchased from California Corporation for Biochemical Research. The imidazole is a product of Eastman Kodak Company. The EGTA,[2] a gift of Geigy Chemical Corporation, New York, New York, was standardized by titration against calcium using calcein (17) as indicator and a Turner fluorometer to follow the titration.

RESULTS

Fig. 1 shows that the percentage of non-resin bound Ca^{45} is increased markedly by the presence of the known calcium chelator EGTA and fortuitously to almost the same extent by muscle preparation P-3, which was being tested for the presence of a soluble calcium-binding substance. The control solution,

[2] EGTA: 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane.

which was prepared, see legend to Fig. 1, in the manner used to produce the hypothetical soluble relaxing factor (12) showed no evidence of the presence of a calcium-binding substance, for the percentage of Ca^{45} bound to resin remained constant over the complete range of calcium concentrations studied. This nearly constant distribution of Ca^{45} was expected since the concentration of resin calcium-binding sites (approximately $1.2 \cdot 10^{-2}$ M, see later section) was considerably higher than all calcium concentrations studied. The

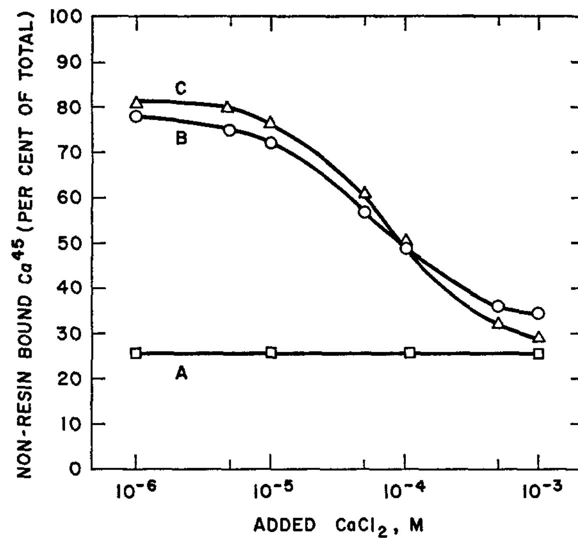


FIGURE 1. Influence of EGTA and the soluble muscle fraction P-3 on the binding of Ca^{45} to chelex-100. Conditions of assay: 50 mM KCl, 30 mM imidazole, 4 mM $MgCl_2$, 4 mM ATP, 1 mM K oxalate, 0.26 ml chelex-100, pH 6.7, total volume 5.26 ml. Curve *A* illustrates Ca^{45} binding in the control solution. This solution was prepared by incubating solution III at room temperature with heavy microsomes (0.25 ml/10 ml of solution III) for 10 minutes and then centrifuging the mixture at 198,000 g for 60 minutes. Curve *C*, a solution identical to that shown in curve *A*, except that EGTA was added to give a concentration of $4.2 \cdot 10^{-5}$ M. Curve *B*, muscle preparation P-3.

presence of the calcium sequestering agents, EGTA and P-3, was most apparent at low calcium concentrations where they competed successfully with the resin for Ca^{45}. As the calcium concentration was raised the sequestering agents became saturated and the resin bound more nearly its control percentage of the Ca^{45}. The range of calcium concentrations over which the percentage of Ca^{45} bound to the resin was increasing is related both to the calcium stability constant and concentration of the calcium sequestering agent, though more to the latter than the former. Since it would be impossible to duplicate the EGTA curve by any combination of concentrations and calcium stability constants very different from those used to produce the EGTA curve one may

conclude that the muscle preparation P-3 must have contained a calcium-binding substance in a concentration of about $5 \cdot 10^{-5}$ M and characterized as having a calcium stability constant similar to that of EGTA.

Muscle preparation P-3 contains both ATP and oxalate and it was of interest to determine if either of these substances are involved in the calcium sequestration. Fig. 2 shows the calcium sequestering action of muscle preparations free of ATP, P-2; and free of ATP and oxalate, P-1. Quite obviously neither ATP nor oxalate are involved in the calcium sequestering action of these soluble muscle fractions.

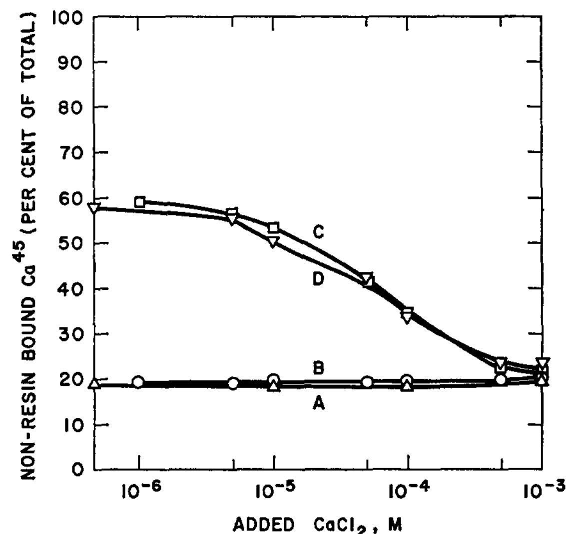


FIGURE 2. Influence of muscle fractions free of oxalate and ATP on the binding of Ca^{45} by chelex-100. Curve *A*, solution I; curve *B*, solution II; curve *C*, muscle preparation P-2, curve *D*, muscle preparation P-1. Solution volume 5 ml, resin volume 0.26 ml.

In order to calculate the concentration and calcium stability constant of the calcium-binding substance in P-1, P-2, and P-3 it was necessary to determine the concentration and calcium stability constant of the resin chelex-100. This was done with the aid of the data illustrated in Fig. 3. Curve *B* shows the percentage of soluble Ca^{45} in solution II when resin is absent. This curve has been used to correct for the formation of insoluble calcium oxalate precipitate which would otherwise be confused with the insoluble calcium resin complex. When applying this data to the system shown in curve *A* it has been assumed that the resin bound about 50 per cent of the calcium so that the concentration of calcium involved in the formation of calcium oxalate was only one half the total calcium concentration. Since the magnitude of the calcium oxalate precipitate was slight the inaccuracy of this correction does

not play a large role in future calculations. It should also be noted that at calcium concentrations below $1 \cdot 10^{-3}$ M no correction need be made. Thus

$$\text{Ca}Ox_{\text{ppt}} = \text{Ca}_T \times \frac{(100 - \text{per cent soluble Ca}^{45})}{100} \qquad (1)$$

where Ox = oxalate, Ca_T = total calcium concentration and per cent soluble Ca^{45} was read from curve *B* at a concentration one-half that for which the correction is to be made.

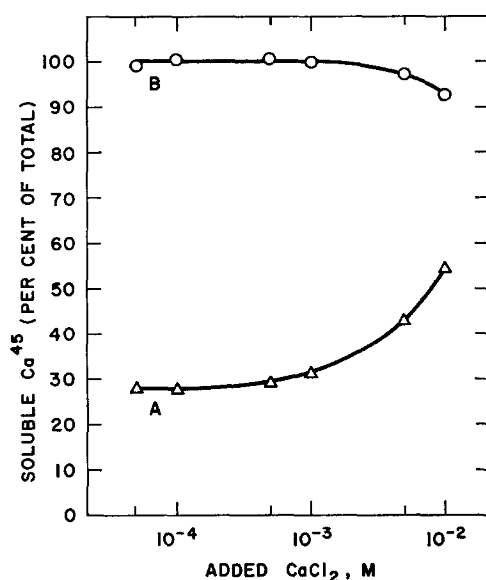
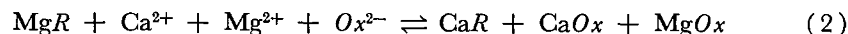


FIGURE 3. Ca^{45} binding by chelex-100 at different calcium concentrations. Assay medium; 50 mM KCl, 30 mM imidazole, 4 mM MgCl_2, 1 mM K oxalate, pH 6.7. Curve *A*, 0.13 ml chelex-100 in a total volume of 5.13 ml. curve *B*, no addition and a total assay volume of 5.0 ml.

Curve *A* shows the percentage of Ca^{45} which was soluble in solution II in the presence of the resin, chelex-100. In this experiment the concentration of the resin was decreased by 50 per cent from that employed in the experiments shown in Figs. 1 and 2. With the data from curve *A* the concentration of calcium-binding sites, R_T, and the apparent calcium stability constant $\text{K}'_{\text{Ca}R}$ were calculated, using molar concentrations for all expressions. The following equilibrium is assumed to pertain.



$$\text{Mg}R + \text{Ca}^{2+} + \text{Mg}^{2+} + Ox^{2-} \rightleftharpoons \text{Ca}R + \text{Ca}Ox + \text{Mg}Ox \qquad (2)$$

The Ca^{45} is divided as follows

$$\text{Ca}_T = \text{Ca}R + \text{Ca}Ox_{\text{ppt}} + \text{Ca}Ox + \text{Ca}^{2+} \qquad (3)$$

$CaOx_{ppt}$ may be calculated from equation (1) and $CaOx + Ca^{2+}$ from

$$CaOx + Ca^{2+} = (Ca_T - CaOx_{ppt}) \frac{(\text{per cent } Ca^{45} \text{ soluble})}{100} \quad (4)$$

CaR may then be calculated from equations (1), (3), and (4).

Since the binding of calcium to resin displaces magnesium and the resin has been saturated with magnesium during preparation, we may write that the final concentration of $Mg^{2+} + MgOx$ will equal the initial concentration of these two species plus that magnesium released by the formation of CaR, the calcium–chelex-100 complex, so that

$$(Mg^{2+} + MgOx)_{final} = (Mg^{2+} + MgOx)_{initial} + CaR \quad (5)$$

Also

$$Ox_T = CaOx_{ppt} + CaOx + MgOx + Ox^{2-} \quad (6)$$

The stability constants for calcium and magnesium oxalate give

$$MgOx = 450\,(Mg^{2+})\,(Ox^{2-}) \quad (7)$$

and

$$CaOx = 160\,(Ca^{2+})\,(Ox^{2-}) \quad (8)$$

The stability constant for magnesium oxalate was taken from Martell and Calvin (18) using Simms' data at $\mu = 0.07$. The calcium-oxalate stability constant was calculated for $\mu = 0.07$ from that given by Martell and Calvin for $\mu = 0$ by use of Simms' data.

Equations (4) through (8) were solved simultaneously by a graphical method (19) to give Ca^{2+} at each Ca_T concentration. The results of these calculations are given in Table I.

The apparent calcium–chelex-100 stability constant

$$K'_{CaR} = \frac{CaR}{(Ca^{2+})(R_T - CaR)} \quad (9)$$

contains an expression for the total concentration of calcium-binding sites (R_T). Since K'_{CaR} is independent[3] of total calcium concentration and CaR and Ca^{2+} are known for various total calcium concentrations; Table I, expression (10) may be used to solve for R_T.

[3] It is assumed that K'_{CaR} does not change with changing Mg^{2+}. Evidence in support of this assumption is given in Appendix II.

$$\left\{\frac{\mathrm{Ca}R}{(\mathrm{Ca}^{2+})(\mathrm{R}_T - \mathrm{Ca}R)}\right\}_i = \left\{\frac{\mathrm{Ca}R}{(\mathrm{Ca}^{2+})(R_T - \mathrm{Ca}R)}\right\}_{ii} \tag{10}$$

The subscript i indicates that the values on this side of equation (10) are taken at one total calcium concentration and ii indicates that the values on

TABLE I

CONCENTRATIONS OF COMPONENTS OF SOLUTION II AT VARIOUS TOTAL CALCIUM CONCENTRATIONS (FIG. 3)

Ca_T	$\mathrm{Ca}Ox^{1*}$ ppt	$\mathrm{Ca}R^{3}$	$\mathrm{Mg}^{2+} + \mathrm{Mg}Ox^{5}$	$\mathrm{Ca}^{2+} + \mathrm{Ca}Ox^{4}$	$\mathrm{Ca}^{2+\,4,5,6,7,8}$
mM	*mM*	*mM*	*mM*	*mM*	*mM*
0.05	0	0.0353	4.03	0.014	0.0128
0.10	0	0.0717	4.07	0.028	0.0255
0.50	0	0.357	4.37	0.14	0.129
1.00	0	0.680	4.68	0.31	0.286
5.00	0.16	2.69	6.69	2.15	2.06
10.00	0.29	4.47	9.62	5.24	5.12

* Superscripts identify the equation used. Curve B of Fig. 3 displays the data used for these calculations.

TABLE II

CONCENTRATION OF CHELEX-100 CALCIUM-BINDING SITES ($\mathrm{R_T}$)

Ca_T		
i	ii	R_T
	M	*M*
$5 \cdot 10^{-4}$	$5 \cdot 10^{-3}$	$4.80 \cdot 10^{-3}$
$5 \cdot 10^{-4}$	$1 \cdot 10^{-2}$	$6.35 \cdot 10^{-3}$
$1 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	$5.15 \cdot 10^{-3}$
$1 \cdot 10^{-3}$	$1 \cdot 10^{-2}$	$6.69 \cdot 10^{-3}$
$5 \cdot 10^{-3}$	$1 \cdot 10^{-2}$	$8.07 \cdot 10^{-3}$
		Average $6.21 \cdot 10^{-3}$

The data used for these calculations were taken from Table I. For the meaning of i and ii see text.

this side of the expression are taken at another total calcium concentration. Values for R_T so obtained are given in Table II.

The average value of R_T was substituted into equation (9) to calculate $K'_{\mathrm{Ca}R}$. The results from these calculations are given in Table III.

Knowing the parameters R_T and $K'_{\mathrm{Ca}R}$ of the resin it is possible to calculate both the concentration and apparent calcium stability constant of a calcium sequestering agent by measuring, as done in Fig. 1, the partition of Ca^{45} between bound and soluble forms. Shown below is a general solution to this

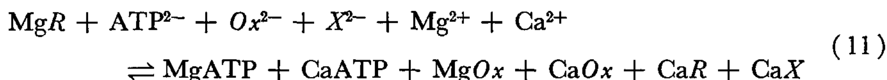
problem which was applied first to the calculation of the concentration and stability constant of a known calcium chelator, EGTA and then to the muscle preparations P-1, P-2, and P-3. The derivation assumes that there is only one calcium sequestering species. The success of the calculations at all total calcium concentrations suggests that the dominant calcium sequestering agent(s) are behaving as single species, though the data are equally applicable to a number of species with very similar stability constants.

TABLE III
ESTIMATES OF THE APPARENT CALCIUM-CHELEX-100 STABILITY CONSTANT

Calcium concentration	Apparent stability constant, K'
M	M^{-}
$5 \cdot 10^{-5}$	$4.7 \cdot 10^{2}$
$1 \cdot 10^{-4}$	$4.6 \cdot 10^{2}$
$5 \cdot 10^{-4}$	$4.7 \cdot 10^{2}$
$1 \cdot 10^{-3}$	$4.3 \cdot 10^{2}$
$5 \cdot 10^{-3}$	$3.7 \cdot 10^{2}$
$1 \cdot 10^{-2}$	$5.3 \cdot 10^{2}$
	Average $4.6 \cdot 10^{2}$

The data used for these calculations were taken from Tables I and II.

The following equilibrium describes the experimental conditions pertaining in Fig. 1.



$$\begin{aligned} &\mathrm{Mg}R + \mathrm{ATP}^{2-} + Ox^{2-} + X^{2-} + \mathrm{Mg}^{2+} + \mathrm{Ca}^{2+} \\ &\quad \rightleftharpoons \mathrm{MgATP} + \mathrm{CaATP} + \mathrm{Mg}Ox + \mathrm{Ca}Ox + \mathrm{Ca}R + \mathrm{Ca}X \end{aligned} \quad (11)$$

where X is any calcium-sequestering agent.

The concentration of CaR, the calcium chelex-100, complex is

$$\mathrm{Ca}R = \mathrm{Ca}_T \frac{(100 - \text{per cent Ca}^{45} \text{ not bound})}{100} \quad (12)$$

The concentration of the sequestered calcium, may be obtained from

$$\mathrm{Ca}X = \mathrm{Ca}_T - \frac{\mathrm{Ca}R}{f} \quad (13)$$

an expression derived in Appendix I. The value of f is taken from curve A of Fig. 1 and is

$$f = \frac{100 - \text{per cent Ca}^{45} \text{ not resin bound}}{100} \quad (14)$$

The total concentration of the calcium-sequestering agent, X_T, can be calculated from an expression equivalent to equation (10) where X is substituted for R in the expression. The value of K'_{CaX} can be obtained from

$$K'_{CaX} = \frac{(CaX)(K'_{CaR})(R_T - CaR)}{CaR(X_T - CaX)} \qquad (15)$$

The above method of calculation has been tested by applying it to the EGTA data in Fig. 1, a case in which the calculated concentration and stability constant can be compared to known or published values. The calculated values are shown in Table IV. The estimated value of $EGTA_T$, $4.6 \cdot 10^{-5}$

TABLE IV
STABILITY CONSTANT AND CONCENTRATION OF EGTA
(from data in Fig. 1)

Experiment	Ca_T	CaR	CaEGTA	$EGTA_T$ i	$EGTA_T$ ii	$EGTA_T$	K'_{CaEGTA}
	M	M	M			M	M^{-1}
a	$1 \cdot 10^{-6}$	$2.39 \cdot 10^{-7}$	$6.78 \cdot 10^{-7}$	c	d	$3.59 \cdot 10^{-5}$	$3.44 \cdot 10^{5}$
b	$5 \cdot 10^{-6}$	$1.24 \cdot 10^{-6}$	$3.33 \cdot 10^{-6}$	c	e	$4.13 \cdot 10^{-5}$	$3.53 \cdot 10^{5}$
c	$1 \cdot 10^{-5}$	$3.04 \cdot 10^{-6}$	$5.90 \cdot 10^{-6}$	c	f	$6.33 \cdot 10^{-5}$	$2.74 \cdot 10^{5}$
d	$5 \cdot 10^{-5}$	$2.16 \cdot 10^{-5}$	$2.09 \cdot 10^{-5}$	c	g	$4.07 \cdot 10^{-5}$	$2.14 \cdot 10^{5}$
e	$1 \cdot 10^{-4}$	$5.15 \cdot 10^{-5}$	$3.05 \cdot 10^{-5}$	d	e	$4.55 \cdot 10^{-5}$	$2.10 \cdot 10^{5}$
f	$5 \cdot 10^{-4}$	$3.46 \cdot 10^{-4}$	$5.33 \cdot 10^{-5}$	d	f	$6.30 \cdot 10^{-5}$	—
g	$1 \cdot 10^{-3}$	$7.12 \cdot 10^{-4}$	$4.00 \cdot 10^{-5}$	d	g	$4.13 \cdot 10^{-5}$	
				e	f	$6.10 \cdot 10^{-5}$	
				e	g	$4.03 \cdot 10^{-5}$	
				f	g	$3.30 \cdot 10^{-5}$	
					Average	$4.65 \cdot 10^{-5}$	$2.8 \cdot 10^{5}$

M, compares favorably with its known concentration, $4.2 \cdot 10^{-5}$ M. The apparent stability constant, $2.8 \cdot 10^{5}$ M^{-1} is definitely lower than the literature values of $6.6 \cdot 10^{5}$ M^{-1} (20) and $1.32 \cdot 10^{6}$ M^{-1} (21). The composition of the solution used in this experiment makes it difficult to use the literature values for comparison. It is noteworthy that the literature values vary by a factor of 2.

The stability constant and concentration of calcium-binding substance in the muscle preparation P-3 has been calculated by the procedure derived above. The results are given in Table V. As expected from the appearance of the curve, the concentration of calcium-binding substance, $9.2 \cdot 10^{-5}$ M, is greater than EGTA and the stability constant, $1.15 \cdot 10^{5}$ M^{-1}, lower than the EGTA constant. The data shown in Fig. 2 have been used to calculate the

concentration and stability constant of preparations P-2 and P-1. They give a concentration of $3.9 \cdot 10^{-5}$ M and a K′ of $1.24 \cdot 10^5$ M^{-1} for P-2 and a concentration of $2.8 \cdot 10^{-5}$ M and a K′ of $2.16 \cdot 10^5$ M^{-1} for P-1. The calcium-complexing substance from muscle thus appears to have a calcium stability constant about 1/2 that of EGTA at pH 6.7. Its concentration in muscle would be 4 times greater than in the preparations studied since the muscle substance has been diluted by this factor during its preparation. Its concentration in muscle would therefore, be approximately $2 \cdot 10^{-4}$ M.

The chemical nature of the calcium-binding substance has not been extensively studied to date. It does, however, appear to be a protein. Thus cold

TABLE V

SUMMARY OF CALCULATED CONCENTRATION AND CALCIUM STABILITY CONSTANTS OF CALCIUM-COMPLEXING SUBSTANCES

Calcium-complexing system	Calculated concentration	Calculated apparent stability constant
	M	M^{-1}
EGTA	$4.6 \pm 0.4^* \times 10^{-5}$ (10)	$2.8 \pm 0.3 \times 10^5$ (5)
P-1	$2.8 \pm 0.3 \times 10^{-5}$ (6)	$2.2 \pm 0.1 \times 10^5$ (5)
P-2	$3.9 \pm 0.6 \times 10^{-5}$ (6)	$1.2 \pm 0.1 \times 10^5$ (5)
P-3	$9.2 \pm 1.2 \times 10^{-5}$ (10)	$1.2 \pm 0.2 \times 10^5$ (6)

* Standard error of the mean, $(\Sigma(\bar{x} - x)^2)/[N(N - 1)]$; ( ) number of observations.

5 per cent trichloracetic acid was found to quantitatively precipitate the calcium-binding activity. On the other hand it is not likely to be a nucleic acid since muscle preparation P-1 when digested in hot perchloric acid yielded no detectable phosphate when tested by the King (22) phosphomolybdate method. The molecular weight of the calcium-binding substance is unknown but must be greater than 4000 for it was not retarded on a sephadex G-25 column.

Two other tissues have been examined for the presence of a calcium-binding substance like that isolated from rabbit skeletal muscle. When muscle fraction P-1 was prepared from dog myocardium a similar calcium-binding substance was detected. When a fraction similar to P-1 was prepared from rabbit liver, using a muscle microsome preparation for the removal of contaminating

calcium, no calcium-binding substance with activity like that from skeletal muscle was detected.

DISCUSSION

Fuchs and Briggs (6, 14) noting that a soluble supernatant fraction of muscle produced relaxation in a manner apparently identical to that produced by the soluble muscle-relaxing factor (12–14) postulated that these two types of preparations might produce their effects by identical mechanisms. The data presented in this report clearly show that this is not the case. Fractions P-1, P-2, and P-3 which are similar in nature to the preparation studied by Fuchs and Briggs (9) contain a calcium-binding substance, while the control solution, prepared in a manner identical to that used to make soluble muscle-relaxing factor, contained no calcium-binding substance. The report of Seidel and Gergely (15) makes it clear that the muscle relaxation observed with the soluble muscle-relaxing factor is due to intrinsic lack of calcium. The soluble muscle supernatant fraction on the other hand could produce relaxation in calcium-containing systems by the sequestration of calcium.

The relaxing activity of supernatant fractions of muscle has apparently been confirmed by Baltchefsky (10) and by Elison (11) but denied in a brief report by Lorand (23). Since few of the experimental details appear in the report by Lorand it is difficult to judge possible reasons for such a discrepancy. The general importance of preparative procedures to the outcome may, however, be relevant to Lorand's results. During the preparation of a muscle homogenate calcium is most likely released and would obscure the presence of a calcium-binding substance unless withdrawn by the microsome fraction before its removal from the homogenate. Microsomes and ATP have been added during the preparation of P-1, P-2, and P-3 to assure the removal of such calcium. The procedure followed by Lorand may not have made this possible.

The most important consequence of the work on muscle-relaxing factors has been the recognition that calcium is the regulator of the activity of actomyosin containing systems and that such materials as muscle microsomes, mitochondria, and soluble supernatant fractions appear to exert their influence upon actomyosin systems indirectly, through influences upon calcium concentration. To be resolved is the physiological role of each of these materials in the regulation of muscle contraction. The theoretical guidelines for an answer are clear. The substance of importance will be the one in which calcium binding is itself subject to regulation so that it is reversible. Evidence that sarcolemma Ca^{45} flux rates change during repetitive stimulation of muscle has been presented by Bianchi and Shanes (24). These flux rates were insufficient, however, to allow the activation of more than a few per cent of the myosin ATPase sites (25). Recent studies by Winegrad (26) suggests that

there is a translocation of intracellular calcium from the middle of the I band into the area of the A-I junction as a result of potassium-induced contracture. The magnitude of the calcium translocation appeared sufficient to account for the activation of a major proportion of myosin sites.

None of the information concerning the nature of the calcium-binding protein suggests that its calcium stability constant is subject to change. The fact that its binding activity is independent of ATP suggests that it is a purely physicochemical activity. What then is the physiological relevance of the calcium-binding protein? Since the answer to this question depends upon where in the cell the calcium-binding substance is, and this is unknown, it is only possible to outline some of the consequences which can be predicted for

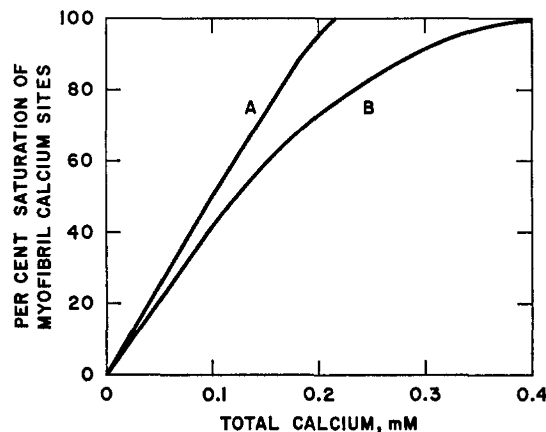


FIGURE 4. Influence of the calcium-binding substance on the amount of calcium required to activate contraction in the myofibrils in one kg of muscle. These are theoretical curves based on assumptions given in the text. Curve *A*, myofibrils. Curve *B*, myofibrils in the presence of calcium-binding protein.

a number of different intracellular distributions. If the calcium-binding substance is derived from the sarcoplasm then it would function there as a calcium buffer system. This buffer system would constitute a calcium sink in competition with the myofibrils for the calcium released, presumably from the sarcotubular system, during the activation of contraction. The consequence of this competition may be predicted if the concentration and stability constants of the calcium-binding protein and myofibril calcium sites are known. Although these data are not precisely known, enough information is available to allow some estimate of the effect. Fig. 4 shows the binding of calcium to myofibrils in the absence of calcium-binding protein, curve *A*, and in the presence of calcium-binding protein, curve *B*. These calculations are based on the following assumption: the concentration of myofibril calcium-binding sites in muscle is 0.2 mM (2) and the calcium stability constant

is $2 \cdot 10^6$ M^{-1} (Fig. 1 of reference 2) and the concentration of calcium-binding sites on the calcium-binding protein is 0.2 mM in muscle and its calcium stability constant is $2 \cdot 10^5$ M^{-1} as reported in this communication. It is quite apparent from Fig. 4 that the presence of the calcium-binding protein about doubles the amount of calcium which must be released in order to fully activate contraction.

The calcium-binding protein, instead of being in the sarcoplasm, might be associated in a number of ways with the calcium binding or calcium uptake by the sarcotubule system. Thus it might play a role in the binding of calcium to the vesicular membrane (27) or it might be involved as a carrier in the transport of calcium across the membrane. To rapidly drop the level of ionic calcium to the level required to suppress contraction would require a carrier with a substantial binding constant for calcium. The value of this constant has been estimated by Ebashi (28) to be about $4 \cdot 10^6$. Finally, the protein might function as an intravesicular binding site for calcium. This would be of considerable advantage for the storage of calcium in the sarcotubular system, for it should decrease the substantial afflux rate which exists in the absence of some calcium inactivation mechanism (29). This, of course, would be a disadvantage when it came time to release calcium in response to an action potential.

A third possible intracellular origin of the calcium-binding substance might be the myofibril. As mentioned earlier Weber's (2) data suggest that the myofibril calcium stability constant is very high. Although the calcium stability constants for the various myofibrillar proteins have not been extensively studied it appears that none of the known proteins possess this type of calcium stability constant. The calcium stability constant of myosin appears (30) to be several orders of magnitude too low. The calcium bound to actin on the other hand is not exchangeable (31). Tropomyosin is an attractive candidate for the calcium-binding site on the myofibril since a protein resembling tropomyosin when mixed with purified actomyosin has been shown by Ebashi (32) to render actomyosin sensitive to control by calcium. Preliminary experiments in our laboratory suggest that trypomyosin does not have a large calcium stability constant. Thus none of the known proteins of the myofibril appears to possess a calcium stability constant of sufficient magnitude to account for the calcium-binding activity of actomyosin. The calcium-binding substance discussed in this communication comes the closest to having the appropriate calcium stability constant. It is possible, therefore, that the calcium-binding substance discussed here is associated with the myofibril. On the other hand, a combination of proteins may give the myofibril its high calcium binding constant.

Elison *et al.* (11) have recently presented evidence which they interpret to indicate that the relaxing effects of a dialyzed muscle supernatant are not

attributable to a calcium-binding agent therein, a conclusion directly opposite of ours. Since the concentration of the calcium buffer system used by Elison was $2.5 \cdot 10^{-4}$ M, or higher, it is possible that the presence of a calcium-binding substance at a concentration of $5 \cdot 10^{-5}$ M, as measured in our supernatant, might not have been detected. Furthermore, the data which they present in favor of the hypothesis that a supernatant fraction of muscle produces relaxation without changing the level of ionic calcium are subject to the criticism that the experiments were carried out with glycerinated muscle bundles rather than single fibers (25). That this criticism may be valid is suggested by the slight relaxation, 27 per cent, observed by Elison at an ionic calcium concentration of $1 \cdot 10^{-7}$ M, a relaxation which is much lower than that observed by Weber and Herz (2) and Seidel and Gergely (3) at the same levels of ionic calcium. Briggs and Portzehl (25) observed that fiber bundles such as used by Elison do not relax normally unless a transphosphorylation system is added. Since myokinase would be a constituent of a muscle supernatant preparation its addition in Elison's study may have merely revealed the expected relaxation rather than causing relaxation.

Appendix I

Examination of curve A of Fig. 1 shows that over the range of calcium concentrations studied the calcium resin concentration, CaR, is a nearly constant percentage of the total calcium concentration, Ca_T *i.e.*,

$$\frac{\mathrm{Ca}R}{\mathrm{Ca}_T} = f \qquad (16)$$

Under the conditions prevailing for curve A, we may write the following expression which says that the total calcium concentration is equal to the sum of the various calcium-containing substances plus ionic calcium,

$$\mathrm{Ca}_T = \mathrm{Ca}R + \mathrm{CaATP} + \mathrm{Ca}Ox + \mathrm{Ca}^{2+} \qquad (17)$$

We may then define the following stability constants:

$$K_1 = \frac{\mathrm{Ca}\,R}{(\mathrm{Ca}^{2+})(R^{2-})}; \qquad \mathrm{Ca}^{2+} = \frac{(\mathrm{Ca}\,R)}{(K_1)(R^{2-})} \qquad (18)$$

$$K_2 = \frac{\mathrm{CaATP}}{(\mathrm{Ca}^{2+})(\mathrm{ATP}^{2-})}; \qquad \mathrm{CaATP} = K_2(\mathrm{Ca}^{2+})(\mathrm{ATP}^{2-}) \qquad (19)$$

$$K_3 = \frac{\mathrm{Ca}Ox}{(\mathrm{Ca}^{2+})(Ox^{2-})}; \qquad \mathrm{Ca}Ox = K_3(\mathrm{Ca}^{2+})(Ox^{2-}) \qquad (20)$$

substituting expressions (19) and (20) into expression (17) one may write

$$\mathrm{Ca}_T = \mathrm{Ca}R + \mathrm{Ca}^{2+}\ \{(K_2(\mathrm{ATP}^{2-}) + K_3(Ox^{2-}) + 1\} \qquad (21)$$

substituting expression (18) for Ca^{2+}

$$Ca_T = CaR + \frac{CaR}{K_1(R^{2-})}\{(K_2(ATP^{2-}) + K_3(Ox^{2-}) + 1\} \quad (22)$$

rearranging terms

$$\frac{CaR}{Ca_T} = \frac{K_1(R^{2-})}{K_1(R^{2-}) + K_2(ATP^{2-}) + K_3(Ox^{2-}) - 1} \quad (23)$$

Thus,

$$f = \frac{K_1(R^{2-})}{K_1(R^{2-}) + K_2(ATP^{2-}) + K_3(Ox^{2-}) + 1} \quad (24)$$

This expression will remain nearly constant, as it does in curve C, as long as R^{2-}, ATP^{2-}, and Ox^{2-} are changing very little. This condition is met as long as the calcium concentration is low.

If now a calcium-binding agent X is present, equation 17 may be rewritten

$$Ca_T = CaR + CaX + CaATP + CaOx + Ca^{2+} \quad (25)$$

or

$$Ca_T - CaX = CaR + CaATP + CaOx + Ca^{2+} \quad (26)$$

The right side of equation (26) is equal to the right side of equation (17). Therefore,

$$\frac{CaR}{Ca_T - Ca\,X} = \frac{K_1(R^{2-})}{K_1(R^{2-}) + K_2(ATP^{2-}) + K_3(Ox^{2-}) + 1} \quad (27)$$

The right side of equation (27) is equal to equation (23). Therefore,

$$\frac{CaR}{Ca_T - Ca\,X} = f \quad (28)$$

or

$$Ca\,X = Ca_T - \frac{CaR}{f} \quad (29)$$

Appendix II

It is assumed in equation (10) and in other parts of this article that K'_{CaR} does not change with changing values of Mg^{2+}. By comparing theoretical and observed ratios of CaR/Ca_T for the experiments reported in Figs. 1 and 2, which represent conditions

with widely different Mg^{2+}, the essential constancy of K'_{CaR} under these conditions can be verified.

Under the conditions depicted for curve A of Fig. 2 the value of Mg^{2+} is 4 mM. The theoretical value of the ratio CaR/Ca_T for this curve may be calculated from the following equation:

$$\frac{CaR}{Ca_T} = \frac{K_1(R^{2-})}{K_1(R^{2-}) + 1} \tag{30}$$

For the meaning of symbols and the derivation of this equation, see Appendix I.

Since $K_1 = 4.6 \cdot 10^2$ M^{-1} (Table III) and $R^{2-} = 2 \times 6.21 \cdot 10^{-3}$ M (Table II), at low calcium concentrations, the theoretical value of $CaR/Ca_T = 0.85$. The observed value of the ratio, 0.81, is in reasonable agreement with the theoretical value.

Fig. 1 represents a condition where Mg^{2+} is greatly reduced below the value of 4 mM. In these experiments ATP was 4 mM and oxalate 1 mM; since both of these substances bind Mg^{2+} its concentration would be very small. It is, therefore, important to see how this affects the value of K'_{CaR}.

For curve A, Fig. 1,

$$\frac{CaR}{Ca_T} = \frac{K_1(R^{2-})}{K_1(R^{2-}) + K_2(ATP^{2-}) + K_3(Ox^{2-}) + 1} \tag{31}$$

For low values of Ca_T, $R_2^- = 1.24 \cdot 10^{-2}$ M. If K'_{CaR} is not substantially altered by the large reduction of Mg^{2+} then $K_1 = 4.6 \cdot 10^2$ M^{-1}. The value of K_2 may be calculated from the following:

$$\text{True } K_{CaATP} = K_2[1 + (H)(K_H) + (Mg)(K_{Mg}) + K^+(K_{K^+})] \tag{32}$$

True K_{CaATP} = $9.4 \cdot 10^3$ M^{-1}, reference (33).

H = $2 \cdot 10^{-7}$ M

K_H = $3.2 \cdot 10^6$ M^{-1}, reference (18).

Mg = $4 \cdot 10^{-3}$ M

K_{Mg} = $1.66 \cdot 10^4$ M^{-1}, reference (33).

K^+ = $5 \cdot 10^{-2}$ M

K_{K^+} = $1.1 \cdot 10'$ M^{-1}, reference (33).

Substituting these values into equation (32) $K_2 = 1.36 \cdot 10^2$. Since $ATP^{2-} = 4 \cdot 10^{-3}$ M, $K_3 = 1.6 \cdot 10^2$, and $Ox^{2-} = 1 \cdot 10^{-3}$ M, $CaR/Ca_T = 0.77$. This compares favorably with the observed value 0.74. Thus K'_{CaR} does not appear to be significantly

dependent upon Mg^{2+} over the range of values encountered in the experiments reported in this paper.

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