*Biochimica et Biophysica Acta*, 449 (1976) 304–309


BBA 47181

# EFFECTS OF IONIC STRENGTH ON CALCIUM BINDING TO RABBIT SKELETAL MYOFIBRILS, THIN FILAMENTS AND MYOSIN

R. JOHN SOLARO, FRANK D. BRUNI and EDWARD N. GLEASON

*Department of Physiology, Medical College of Virginia, Richmond, Virginia 23298, (U.S.A.)*

(Received April 7th, 1976)

## SUMMARY

Calcium binding by rabbit skeletal myosin, thin filaments and myofibrils was measured in solutions with and without 2 mM MgATP and with ionic strengths adjusted with KCl to 0.05, 0.10 and 0.14 M. Free $Mg^{2+}$ was held constant at 1 mM, pH at 7.0 and temperature at 25 °C. In the presence of MgATP, the relation between free $Ca^{2+}$ and myofibrillar bound calcium shifted to the left as ionic strength was decreased from 0.14 to 0.05 M. In the absence of MgATP, myofibrillar calcium binding was enhanced over a wide range of free $Ca^{2+}$ concentration, but calcium binding was no longer a function of ionic strength. Similarly, calcium binding by thin filaments and myosin was unaffected by changes in ionic strength from 0.05 to 0.14 M. In view of evidence that cross-bridge connections between thick and thin filaments increase as ionic strength decreases, our results suggest that these connections enhance myofibrillar calcium binding. These results thus confirm previous data of Bremel and Weber (Bremel, R. D. and Weber, A. (1972) Nature New Biol. 238, 97–101) who first showed that nucleotide-free cross-bridge connections enhance thin filament calcium binding.

## INTRODUCTION

Decreases in ionic strength (salt concentration) below 0.15 M have well known effects on the activity of myofibrillar proteins. Homsher et al. [1] and Gordon et al. [2] reported that such decreases in ionic strength elevate both resting or baseline tension and total force of skinned muscle fibers. Thames et al. [3] provided evidence that skinned fibers shorten more slowly as ionic strength is decreased from 0.14 M. Decreases in ionic strength have also been shown to increase basal myofibrillar ATPase (free $Ca^{2+}$ concentration $< 10^{-8}$ M) and to cause leftward shifts of the free $Ca^{2+}$-myofibrillar ATPase relation [4, 5]. Kinetic studies of actin activation of heavy meromyosin ATPase [6] indicate that these effects of low ionic strength may be due to increased actin co-factor activity as defined in reciprocal plots of actin concentration vs. acto heavy-meromyosin ATPase; and there is a general consensus among a number of investigators [1–5] that the mechanism by which decreases in ionic strength induce the mechanical and biochemical alterations cited above is related to increases

in the steady-state concentration of cross-bridge connections between thick and thin filaments.

Recently, Bremel and Weber [7] showed that occupation of the thin filament with nucleotide free cross-bridges (rigor linkages) increased calcium binding to the thin filament calcium receptor troponin-C. This effect of cross-bridge connections has important implications with respect to calcium control of myofibrils (see ref. 8 for review), and it is thus of interest to know whether cross-bridge linkages in the presence of MgATP also influence thin filament calcium binding. Because concentrations of linkages between thick and thin filaments can be varied in presence of MgATP by changes in ionic strength, we decided to measure calcium binding to myofibrillar proteins in solutions of varying salt concentration. Results of these studies indicate that changes in ionic strength and presumably concentrations of acto-myosin linkages elicit changes in myofibrillar calcium binding.

## METHODS

*Preparations.* Rabbit back muscle myofibrils were prepared as described by Solaro et al. [9] and Solaro and Shiner [10], except that the myofibrils were washed with 10 pellet vols. of 2 mM ethyleneglycol bis-($\beta$-amino-ethylether)-*N*, *N'*-tetraacetic acid (EGTA), 2 mM $MgCl_2$, 60 mM KCl, and 30 mM imidazole, pH 7.0, to remove contaminant calcium. The pellets were then washed with this same solution without EGTA and finally suspended at a protein concentration [11] of 15–20 mg/ml. I-Z-I brushes, a myosin-free thin filament preparation, were prepared as described by Fukazawa et al. [12], except that final washings were done in 60 mM KCl, 30 mM imidazole, pH 7.0. Myosin was prepared by the method of Kielly and Harrington [13]. Skeletal myosin ATPase was approx. 0 in 2 mM $Mg^{2+}$ and 1–1.5 $\mu$mol/mg per min in 2 mM ethylenediamine tetraacetic acid (EDTA), 0.5 M KCl, 2 mM ATP, 20 mM imidazole, pH 7.0, at 25 °C. I-Z-I filaments had no ATPase with these latter conditions. All preparations were analysed for purity by sodium dodecyl sulphate disc gel electrophoresis using the method of Fairbanks et al. [14]. Thin filaments were myosin-free and myosin preparations were thin-filament free.

*Calcium binding measurements.* Myosin, I-Z-I filament and myofibrillar bound calcium were measured essentially as described by Solaro and Shiner [10]. Incubation mixtures for calcium binding measurements were prepared by adding an aliquot of protein stock (which had been equilibrated with the final desired concentration of salts and buffer) containing $^{45}CaCl_2$ and D-[$^3$H]glucose to preweighed centrifuge tubes containing desired concentrations of $Na_2ATP$, $MgCl_2$, creatine phosphate, creatine phosphokinase, KCl, NaCl, EGTA and $CaCl_2$. The suspension was mixed well and immediately centrifuged at $3000 \times g$ (myofibrils and I-Z-I filaments) or $100\,000 \times g$ (myosin) for 15 to 20 min. The pellets were thoroughly suspended in 50 mM cold carrier and radioactivity was extracted in either 2 % sodium dodecyl sulphate or 1.4 M perchloric acid. Solute space within pellets was calculated from the D-[$^3$H] glucose counts in the pellet. Bound calcium was computed from the protein concentration, total added and exchangeable contaminant calcium and the fraction of bound $^{45}Ca$. Pilot studies showed that glucose did not affect ATPase or binding of calcium and that perchloric acid and sodium dodecyl sulphate caused no quenching in the Beckman liquid scintillation cocktail we used.

Contaminant calcium in protein preparations, buffer, salt, ATP and creatine phosphate stock solutions was determined with an Instrumentation Laboratories Atomic Absorption Spectrophotometer as previously described [5, 10]. Calcium in protein suspensions was solubilized with 2 mM $MgCl_2$, 2 mM EGTA, at pH 7.0. Under these conditions, myofibrils and myosin bind trivial amounts of calcium, and the supernatant fraction obtained following centrifugation at $17\,000 \times g$ contained rapidly exchangeable calcium. Standard and unknown calcium solutions were treated identically and contained 1 % $LaCl_3$. Contaminant calcium in incubation mixtures was 2–5 $\mu$M.

*Calculation of free $Ca^{2+}$ concentration, free $Mg^{2+}$ concentration and ionic strength.* The system of simultaneous equations describing the multiple equilibria in various incubation solutions was solved with a Wang 700 A/B programmable calculator. The ionic strength, pH, temperature, $Na^+$, $Mg^{2+}$, $Ca^{2+}$ and $MgATP^{2-}$ concentrations were specified for each experiment and the program calculated total concentrations of $CaCl_2$, $MgCl_2$, $Na_2$ ATP and KCl to be added to solutions containing constant concentrations of EGTA, imidazole, creatine phosphate and creatine phosphokinase. Constants for EGTA species used in this program have been shown by Godt [15] to be unaffected by ionic strength over the range we studied. Constants for the ATP species were, however, adjusted for ionic strength using plots given by Phillips et al. [16]. At the three ionic strengths studied the following logarithmic binding constants were used: for the binding of $H^+$ to $EGTA^{4-}$, 9.43; for $H^+$ to $HEGTA^{3-}$, 8.85; for $H^+$ to $H_2EGTA^{2-}$, 2.68; for $H^+$ to $H_3EGTA^-$, 2.0; for $Ca^{2+}$ to $EGTA^{4-}$, 10.4; for $Ca^{2+}$ to $HEGTA^{3-}$, 5.32; for $Mg^{2+}$ to $EGTA^{4-}$, 5.21; for $Mg^{2+}$ to $HEGTA^{3-}$, 3.36; for $H^+$ to $HATP^{3-}$, 4.06; for $K^+$ to $ATP^{4-}$, 1.06; and for $Na^+$ to $ATP^{4-}$, 1.16. At ionic strengths 0.05, 0.10 and 0.14, the respective logarithmic binding constants for the binding of $H^+$ to $ATP^{4-}$ were 7.13, 7.04 and 7.03; for $Ca^{2+}$ to $ATP^{4-}$, 4.61, 4.45 and 4.43; for $Ca^{2+}$ to $HATP^{3-}$, 2.67, 2.60 and 2.59; for $Mg^{2+}$ to $ATP^{4-}$, 4.71, 4.56 and 4.54; and for $Mg^{2+}$ to $HATP^{3-}$, 2.81, 2.73 and 2.72. A $pK_a$, for imidazole of 7.07 was included in the calculation.

## RESULTS AND DISCUSSION

Fig. 1 shows the effects of ionic strength on the relation between free $Ca^{2+}$ concentration and myofibrillar bound calcium. At nearly all levels of free $Ca^{2+}$, myofibrillar calcium binding increased as ionic strength was decreased with constant concentrations of 1 mM free $Mg^{2+}$ and 2 mM MgATP. Shifts in the curves shown in Fig. 1 are statistically significant. In three preparations studied, myofibrillar bound calcium at $5 \cdot 10^{-6}$ M free $Ca^{2+}$: (a) decreased significantly ($P < 0.05$, Student's $t$ test) when ionic strength was increased from 0.10 M (mean$\pm$S.D. bound calcium concentration = 2.42$\pm$0.07 $\mu$mol/g protein) to 0.14 M (mean$\pm$S.D. bound calcium concentration = 1.74$\pm$0.05 $\mu$mol/g protein), and (b) increased significantly ($P < 0.05$) when ionic strength was decreased from 0.10 M to 0.05 M (mean$\pm$S.D. bound calcium concentration = 3.13$\pm$0.12 $\mu$mol/g protein). To determine whether we could attribute this dependence of myofibrillar calcium binding on ionic strength to effects of salt concentration on either troponin or myosin calcium binding, we measured calcium binding to isolated preparations of myosin and I-Z-I filaments. Fig. 2 shows that myosin and I-Z-I filament calcium binding measured with conditions identical to

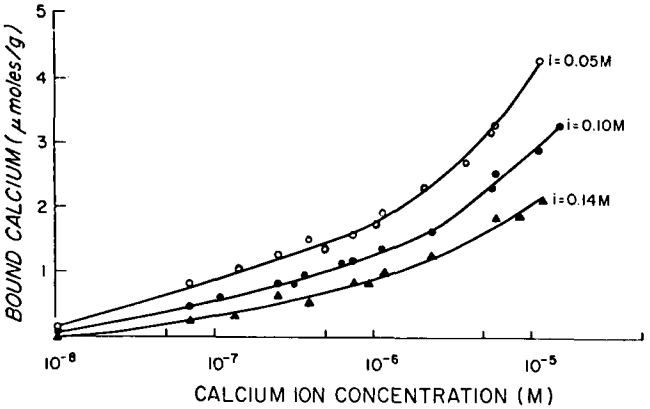


Fig. 1. Effects of ionic strength on myofibrillar calcium binding. Calcium binding by myofibrils (3.0 mg protein/ml) was measured at pH 7.0, 25 °C, 2 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, 20 mM imidazole, 0–300 $\mu$M EGTA, 0–60 $\mu$M $CaCl_2$, 0.5 $\mu$Ci/ml $^{45}CaCl_2$, 0.3 $\mu$Ci/ml D-[$^3$H]glucose. Other conditions were: for $i = 0.05$ M: 3.0 mM $Na_2ATP$, 3.95 mM $MgCl_2$, 3.0 mM KCl, 15.9 mM NaCl; for $i = 0.10$ M: 2.2 mM $Na_2ATP$, 3.0 mM $MgCl_2$, 54.7 mM KCl, 17.6 mM NaCl; for $i = 0.14$ M: 2.5 mM $Na_2ATP$, 3.0 mM $MgCl_2$, 94.7 mM KCl, 17.7 mM NaCl. With these conditions, free $Mg^{2+}$ concentration was held constant at 1 mM and $MgATP^{2-}$ concentration at 2.0 mM.

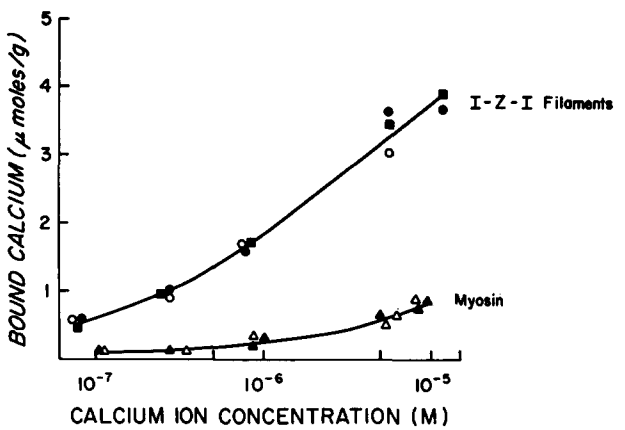


Fig. 2. Effect of ionic strength on calcium binding by I-Z-I filaments and myosin. I-Z-I filament (1.5 mg/ml) calcium binding was measured in ionic strength 0.05 M (○—○), 0.10 M (●—●), and 0.14 M (■—■). Myosin (1.0 mg/ml) calcium binding was measured in ionic strength 0.05 M (△—△) and 0.14 M (▲—▲). Other conditions were as described in Fig. 1.

those of experiments reported in Fig. 1, did not significantly change between ionic strength 0.05 and 0.14. It seems reasonable to conclude therefore that effects of salt concentration on myofibrillar calcium binding were not due to an influence of ionic strength on either thick or thin filament calcium binding alone.

Fig. 3 shows results of experiments in which we measured myofibrillar, myosin and I-Z-I filament calcium binding as functions of free $Ca^{2+}$ concentration

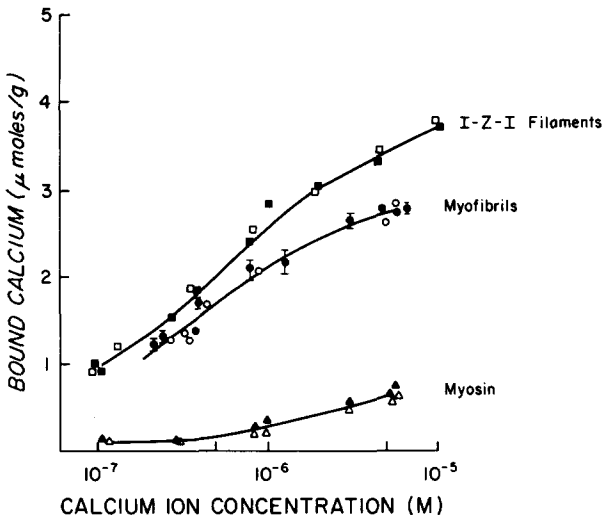


Fig. 3. Effect of ionic strength on calcium binding by I-Z-I filaments, myosin and myofibrils in the absence of MgATP. Conditions were the same as described in Figs. 1 and 2 except that no $Na_2ATP$ was added and added $MgCl_2$ was 1 mM. Ionic strength was adjusted with KCl. I-Z-I filaments ionic strength 0.05 M (□—□) and 0.14 M (■—■). Myofibrils ionic strength 0.05 M (○—○) and 0.14 M (●—●). Myosin ionic strength 0.05 M (△—△) and 0.14 M (▲—▲). Where shown, standard error bars are for 3 experiments.

and ionic strength, but in the absence of MgATP (free $Mg^{2+}$ remained constant at 1 mM). With these conditions, thick filament cross-bridge connection to actin is maximal [17]. Comparison of myofibrillar calcium binding with 2 mM MgATP (Fig. 1) to myofibrillar calcium binding without ATP (Fig. 3) confirms the report of Bremel and Weber [7] who showed that troponin calcium affinity and hence, myofibrillar calcium binding increases in the absence of MgATP. However, as shown in Fig. 3, myofibrillar calcium binding was no longer a function of ionic strength in the absence of MgATP. Presumably, the effect of rigor linkages between actin and myosin to increase troponin calcium affinity is unaffected by ionic strength at least between 0.05 and 0.14 M. Comparison of data in Fig. 2 and Fig. 3 also shows that MgATP had no effect on either myosin or I-Z-I filament calcium binding, and that myosin and I-Z-I filament calcium binding remained independent of ionic strength between 0.05 and 0.14 M in the absence of MgATP. These results agree with previous reports by Bremel and Weber [18] who showed myosin and actin-tropomyosin-troponin calcium binding to be unaffected by MgATP; by Fuchs [19] who showed troponin calcium binding to be independent of ionic strength; and by Morimoto and Harrington [20], who showed myosin calcium binding to be only slightly affected by large changes in KCl concentration.

The most important conclusion of this study is that linkages between actin and myosin in the presence of MgATP alter calcium binding properties of troponin C. We could not account for increased myofibrillar calcium binding by reduction of ionic strength from 0.14 M to 0.05 M to the effects of salt concentration on either myosin or thin filament calcium binding and data reported by Bremel and Weber [18] showed

that myosin calcium binding is unaffected by linkage to actin-tropomyosin. Moreover, we could find no evidence to suggest that changes in ionic strength in the range we studied affect nucleotide binding to myosin or myofibrils. However, we cannot exclude the possibility that linkages between actin and myosin which vary with ionic strength are, like rigor linkages, an aberrant form of cross-bridge connection as discussed by Thames et al. [3]. Nevertheless, it seems clear that cross-bridge connections between actin and myosin formed at low ionic strength affect troponin calcium binding. This implies that myofibrillar calcium control may vary with length changes, speed of shortening and calcium saturation of troponin C, all of which involve changes in the number of connected cross-bridges.

ACKNOWLEDGEMENTS

This work was supported by Grant-in-Aid 74-865 from the American Heart Association and with funds contributed in part from the Richmond Area and Virginia State Heart Associations.

REFERENCES

1 Homsher, E., Briggs, F. N. and Wise, R. M. (1974) Am. J. Physiol. 226, 855–863
2 Gordon, A. M., Godt, R. E., Donaldson, S. K. B., and Harris, C. E. (1973) J. Gen. Physiol. 62, 550–574
3 Thames, M. D., Teichholz, L. E. and Podolsky, R. J. (1974) J. Gen. Physiol. 63, 509–530
4 Portzehl, H., Zaroalek, P. and Gaudin, J. (1969) Biochim. Biophys. Acta 189, 440–451
5 Solaro, R. J. and Briggs, F. N. (1974) in Calcium Binding Proteins (Drabikowski, W., Strzelecka-Golaszewska, H. and Carafoli, E., eds.) pp. 587–607, Elsevier Amsterdam
6 Moos, C. (1973) Cold Spring Harbor Symposia Quant. Biol. 37, 137–143
7 Bremel, R. D. and Weber, A. (1972) Nature (New Biol.) 238, 97–101
8 Weber, A. and Murray, J. M. (1973) Physiol. Rev., 53, 612–673
9 Solaro, R. J., Pang, D. C. and Briggs, F. N. (1971) Biochim. Biophys. Acta 245, 259–262
10 Solaro, R. J. and Shiner, J. S. (1976) Circ. Res. in the press
11 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
12 Fukazawa, T. and Briskey, E. J. (1970) Biochim. Biophys. Acta 205, 328–341
13 Kielly, W. W. and Harrington, W. F. (1960) Biochim. Biophys. Acta 41, 401–421
14 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (19701) Biochemistry 10, 2606–2617
15 Godt, R. E. (1974) J. Gen. Physiol. 63, 722–739
16 Phillips, R. C., George, S. J. P. and Rutman, R. J. (1966) J. Am. Chem. Soc. 88: 12, 2631–2640
17 Huxley, H. H. (1972) Cold Spring Harbor Symp. Quant. Biol., 37, 361–376
18 Bremel, R. D. and Weber, A. (1975) Biochim. Biophys. Acta 376, 366–374
19 Fuchs, F. (1974) Calcium Binding Proteins (Drabikowski, W., Strzelecka-Golaszewska, H., and Carafoli, E., eds.) pp. 1–27, Elsevier, Amsterdam
20 Morimoto, K. and Harrington, W. F. (1973) J. Mol. Biol. 77, 165–175