DIFFERENCES IN REGULATORY MECHANISMS OF ATRIAL AND VENTRICULAR MUSCLE CONTRACTION IN BOVINE HEART

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The purpose of this study was to characterize the regulatory mechanisms of atrial muscle contraction. Natural actomyosin (NAM) and tropomyosin-troponin (TM-TN) complex were prepared from atrial and ventricular muscle of the same bovine heart. The results were as follows: (1) Atrial NAM was more sensitive to Ca^{2+} than was ventricular NAM: the pCa required for 50% ATPase activation was 5.96 ± 0.10 vs. 5.63 ± 0.07 , (mean $\pm$ SE; $n = 6$; $p < 0.01$); (2) reconstitution of desensitized actomyosin of rabbit skeletal muscle plus atrial or ventricular TM-TN complex produced higher Ca^{2+} sensitivity in atrial muscle than in ventricular muscle: the pCa required for 50% ATPase activation was 6.48 ± 0.10 vs. 6.23 ± 0.15 ($n = 3$; $p < 0.05$); (3) the amount of inorganic phosphate covalently bound to atrial NAM was equivalent to that bound to ventricular NAM; (4) SDS-polyacrylamide gel electrophoresis of the two NAMs revealed several protein bands of different mobility from 16,000 to 30,000 daltons; and (5) the superprecipitation response of atrial NAM was characterized by a stepwise change in turbidity after the addition of MgATP, in contrast to the biphasic pattern of ventricular NAM. These data suggest that the free Ca ion concentration required for atrial muscle contraction is lower than that required for ventricular muscle contraction and that the difference is attributable to differences in atrial and ventricular regulatory proteins.

THE tropomyosin-troponin (TM-TN) complex of actin filaments inhibits the interaction between myosin and actin in the absence of free Ca^{2+} (pCa > 7). At micromolar levels of Ca^{2+} , the inhibition is abolished and muscle contracts.^{1,2} These regulatory mechanisms of contraction have been identified in skeletal and cardiac muscle.³⁻⁵

Atrial muscle contractions serve to control

ventricular filling or preload and modify cardiac output. However, despite the physiological significance of atrial activities, previous reports concerning the regulation of cardiac muscle have been restricted to the ventricular tissue.³⁻⁶ The chief purpose of the study we report here was to characterize and compare the regulatory mechanisms of atrial and ventricular muscle, using natural actomyosin (NAM) or TM-TN complexes isolated from the same heart.

Key words:

Regulation mechanism
Natural actomyosin
ATPase
Atrial muscle
Ventricular muscle

MATERIALS AND METHODS

Preparation of NAM

(Received October 14, 1987; accepted January 5, 1988)

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This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture and Science, the Ministry of Health and Welfare, Ca Signal Workshop in Cardiovascular Systems, Mitsukoshi Foundation for Welfare, Shimabara Research Foundation, and Suzuken Memorial Foundation.

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Fresh bovine heart was obtained from a local abattoir. Atrial and ventricular NAM was prepared at below 4°C. The muscles were minced and homogenized in a Waring blender in 6 vol of modified Weber-Edsall's solution (0.6 M KCl, 0.04 M NaHCO₃, 0.01 M Na₂CO₃, 1 mM leupeptin (LP), and 1 mM phenylmethanesulfonyl-fluoride (PMSF)). After the addition of neutralized adenosine 5'-triphosphate (ATP) (final concentration, 2 mM), NAM was extracted overnight. The homogenate was centrifuged at 20,000g for 60 min, and the supernatant was diluted with 6 vol of cold water and centrifuged at 10,000g for 60 min. The precipitate was dissolved in an equal volume of 0.6 M KCl and 10 mM imidazole (pH 7.0) and centrifuged at 20,000g for 20 min; the supernatant was saved. The suspension, centrifugation, and supernatant dilution procedures were repeated twice. Finally, the precipitate was suspended in 0.3 M KCl and 10 mM imidazole buffer (pH 7.0) and kept on ice until use. The ATPase activity was assayed within 3 days of its preparation (n = 6).

Preparation of TM-TN complexes

Atrial and ventricular muscle tissues were homogenized in 5 vol of 30 mM KCl, 20 mM imidazole, 1 mM LP, and 1 mM PMSF at pH 7.0 and centrifuged at 10,000g for 15 min. The pellet was rehomogenized in the same buffer containing 1% Triton X-100 and the precipitate was collected by centrifugation at 10,000g for 15 min. These procedures were repeated twice without Triton X-100. Atrial or left ventricular myofibrils were then mixed with 6 vol of cold acetone and passed through filter paper. The acetone treatment was repeated 4 times. After drying, the TM-TN complex was extracted overnight in 10 vol of 1 M KCl, 5 mM imidazole buffer (pH 7.0), 1 mM LP, and 1 mM PMSF. The extract was centrifuged at 20,000g for 60 min and the supernatant was fractionated with ammonium sulfate between 30 and 60% saturation. The fraction was thoroughly dialyzed with 0.2 mM NaHCO₃ and ultracentrifuged at 100,000g for 2 hr. The resultant supernatant was defined as a TM-TN complex.

Preparation of desensitized actomyosin (DAM)

and reconstitution with TM-TN complex

NAM of rabbit skeletal muscle was prepared as described above, washed in 5 vol of 0.2 mM NaHCO₃, and centrifuged at 20,000g for 20 min. The washing and centrifugation steps were repeated until the pellet had lost its Ca²⁺ sensitivity. The resultant 5 mg of DAM was mixed with 5 mg or less of the TM-TN complex from atrium or left ventricle. The Ca²⁺ dependency of DAM or the reconstituted actomyosin was assayed along with the ATPase activity, as described below.

Measurement of NAM ATPase activity

NAM was preincubated at 30°C for 5 min in 30 mM KCl, 2 mM MgCl₂, 1 mM Ca²⁺-ethylene glycol bis (2-aminoethylether) tetraacetic acid (EGTA) buffer and 50 mM imidazole buffer (pH 7.0), in a final volume of 1 ml. The free Ca²⁺ concentration (pCa), the ionic strength or MgATP concentration was varied as described in each section. The enzyme reaction was started by the addition of ATP (1 mM in final concentration), maintained at 30°C for 10 min, and stopped during the steady state by the addition of 0.5 ml of 20% ice-cold TCA. The liberated inorganic phosphate was quantitated by the Rockstein-Helon method.⁷ All measurements were carried out in duplicate, and the mean was used as the result.

Measurement of protein-bound phosphate

The amount of inorganic phosphate covalently bound to NAM was determined by the procedure of Stull and Buss,⁵ a modification of the inorganic phosphate assay described by Itaya and Ui,⁸ and the ashing method of Ames.⁹ In each assay, the standard curve for phosphate was constructed as 0.1–1 nmol of inorganic phosphate.

Superprecipitation measurement of atrial and ventricular NAM

The superprecipitation response of NAM was assayed under the same conditions as employed for the NAM ATPase activity measurement, according to the method of Ebashi.² The reaction was initiated by the addition of ATP (1 mM in final concentration), and the turbidity of NAM was monitored by spectrophotometry at 660 nm.

Abbreviations: ATP = adenosine 5'-triphosphate; Ca²⁺ = calcium ions; DAM = desensitized actomyosin; LP = leupeptin; MLC = myosin light chain; NAM = natural actomyosin; pCa = -log[free Ca²⁺]; PMSF = phenylmethanesulfonyl fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA = trichloroacetic acid; TM = tropomyosin and TN = troponin.

Fig.1. Effect of Ca^{2+} concentration on left ventricular (solid circles), right ventricular (open circles), and atrial (triangles) NAM ATPase activity. Each point represents the mean $\pm$ SE of six experiments. A: Atrial NAM ATPase showed higher activity than that of left ventricle (* $p < 0.025$; ** $p < 0.01$; *** $p < 0.005$). The inset shows the Hill plot of the ATPase activity. The arrows indicate the pCa required for 50% activation. B: Each point was calculated on the assumption that the ATPase activity at pCa 8 or 4 is 0 or 100%, respectively.

Miscellaneous

All reagents were of analytical grade, and water was redistilled before use. Polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli's method¹⁰ in 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). The protein concentration was determined by the micro-biuret method,¹¹ with bovine serum albumin as a standard. Free Ca^{2+} concentrations were calculated by means of the

apparent binding constant between Ca^{2+} and EGTA determined by Ogawa.¹² Each result was expressed by the mean $\pm$ standard error (SE) and analyzed with Student's paired t-test; p values of less than 5% were considered significant.

RESULTS

Effect of Ca^{2+} on NAM ATPase activity

Figure 1 summarizes the Ca^{2+} dependency of

Fig. 2. Effect of Ca^{2+} concentration on ATPase activity of rabbit skeletal muscle DAM alone (open squares) and DAM with ventricular TM-TN complex (solid circles) or atrial TM-TN complex (triangles). The arrows indicate the pCa required for 50% induction of ATPase activity of each reconstituted actomyosin. Each point represents the mean $\pm$ SE of three experiments, assuming that ATPase activity at pCa 8 and 4 was 0 and 100%, respectively.

Fig. 3. ATPase activity with variable weight ratios of DAM to atrial TM-TN complex. Each point represents the mean ($n = 3$) at pCa 8 (solid circles) or pCa 5 (open circles).

atrial and ventricular NAM ATPase activity. Neither activity changed above pCa 6.8, increased between pCa 6.8 and 5.0, and reached a plateau below pCa 5.0. The pCa required for 50% activation of atrial NAM ATPase (Fig. 1, arrows) was significantly larger than that required for ventricular NAM (5.96 ± 0.10 vs 5.63 ± 0.07 ; $p < 0.01$). Hill's coefficient after probit analysis showed no difference between atrial and ventricular NAM (1.24 ± 0.10 vs 1.09 ± 0.07 ; Fig. 1A, inset).

There was no difference in Ca^{2+} dependency between left and right ventricular NAM. The pCa required for 50% activation was 5.63 ± 0.07 and 5.62 ± 0.06 , respectively (Fig. 1A and B). Hill's coefficient showed no difference in NAM between left and right ventricular muscle (1.09 ± 0.07 vs 1.25 ± 0.07). The findings concerning the dependency of ventricular NAM ATPase activity on pCa were in good agreement with physiological data obtained with glycerine-treated ventricular muscle fibers⁶.

Effect of Ca^{2+} with and without TM-TN complexes on DAM ATPase activity

DAM from rabbit skeletal muscle lost the Ca^{2+} sensitivity and showed constant ATPase activity between pCa 8 and 5 (Fig. 2, squares). After the addition of atrial (triangles) or ven-

Fig. 4. SDS-PAGE of bovine cardiac NAM prepared from atrium (AT), left ventricle (LV), and molecular weight calibration proteins (CAL) (69,000, bovine serum albumin; 45,000, egg albumin; and 18,400, beta-lactoglobulin). MHC, myosin heavy chain; MLC, myosin light chain; and TM, tropomyosin. White arrows denote differences in protein band mobility.

tricular (circles) TM-TN complexes to the DAM, the ATPase sensitivity to Ca^{2+} was restored. As summarized in Fig. 2, the pCa necessary for 50% activation of DAM with atrial TM-TN complex was 6.48 ± 0.10 ($n = 3$), which was larger than that required with left ventricular TM-TN complex (6.24 ± 0.15 ; $p < 0.05$).

When the amount of atrial TM-TN complex added to DAM was altered, the reconstituted DAM exhibited Ca^{2+} sensitivity with TM-TN complex at as low as 1/16 amounts of the DAM (Fig. 3). The maximal inhibitory action of the TM-TN complex was demonstrated at 1/2 amounts of the DAM.

Extent of phosphate binding to NAM

Since phosphorylation of myosin light chains or troponin may influence NAM ATPase activity, we assayed the amount of covalently bound phosphate after complete hydrolysis of the

Fig. 5. SDS-PAGE of TM-TN complex prepared from atrium (AT), ventricle (LV), and molecular weight calibration proteins (CAL) (55,400, pyruvate kinase; 36,500, lactic dehydrogenase; 20,100, trypsin inhibitor). Abbreviations are the same as those in Fig. 4.

phosphate ester-bond⁵ The amounts of atrial and ventricular NAM bound phosphate were 1.01 ± 0.14 ng/mg of protein and 0.66 ± 0.08 ng/mg of protein, respectively. These measurements were performed at least in triplicate. No significant difference was demonstrated between atrial and ventricular NAM.

SDS-PAGE of NAM

To determine the difference in molecular weight between atrial and ventricular contractile proteins, NAMs or TM-TN complexes prepared from both tissues were examined by SDS-PAGE. Ventricular and atrial NAM contained several protein bands having the same rate of mobility, including myosin heavy chains (MHC), actin and myosin light chains 2 (MLC2) (Fig. 4). The molecular weight of myosin light chain 1 was obviously smaller in atrial than in ventricular tissue. Fig. 5 shows the electrophoretogram of the TM-TN complexes from both tissues.

Fig. 6. Effect of ionic strength on NAM ATPase activity of left ventricle (solid circles), right ventricle (open circles), and atrium (triangles). The activity of atrial NAM ATPase was significantly higher than that of ventricular NAM ATPase in the presence of 60 and 120 mM KCl (* $p < 0.025$).

Effect of ionic strength on NAM ATPase activity

NAM ATPase activity was progressively reduced with increasing KCl concentrations at pCa 5 (Fig. 6). The activity of atrial NAM was higher than that of ventricular NAM in the presence of both 60 and 120 mM KCl ($p < 0.025$), whereas they demonstrated the same activity at 240 and 480 mM KCl. The right and left ventricular ATPase showed similar activity regardless of the ionic strength used.

These results were not dependent on the Ca^{2+} concentration. ATPase activity obtained at high (pCa 5.0) and low (pCa 8.0) Ca^{2+} concentrations were similar, although the activity observed at pCa 8.0 was always lower than that seen at pCa 5.0 (data not shown).

Effect of MgATP concentration on NAM ATPase activity

In both atrial and ventricular muscle, MgATP concentrations of 0.5 to 8 mM caused a biphasic response of the ATPase activity at pCa 5 (Fig. 7). Atrial NAM ATPase demonstrated higher activity than did ventricular NAM ATPase in the presence of 2 and 4 mM MgATP ($p < 0.05$ and $p < 0.025$, respectively). At pCa 8, atrial NAM ATPase

Fig. 7. Effects of MgATP concentration on NAM ATPase activity in left ventricle (solid circles), right ventricle (open circles), and atrium (triangles). The activity of atrial NAM ATPase was significantly higher than that of ventricular NAM ATPase in the presence of 2 mM (** $p < 0.025$), 4 mM (** $p < 0.025$), and 8 mM (* $p < 0.05$) MgATP.

displayed the same tendency, although its activity was always lower than that seen at pCa 5 (data not shown).

Superprecipitation response of atrial and ventricular NAM

Figure 8A shows the superprecipitation response of NAM prepared from left ventricle. After addition of ATP (1 mM in final concentration), the turbidity of the NAM immediately dropped, then increased and reached a plateau in the presence of high concentrations of Ca^{2+} (pCa 5.5, 5.7, or 5.9). At lower Ca^{2+} concentrations, the turbidity remained at a reduced level after the addition of ATP.

In contrast, the superprecipitation responses of atrial NAM did not demonstrate a biphasic change. A stepwise increment or reduction of turbidity was observed after the addition of ATP at both high and low free Ca^{2+} concen-

Fig. 8. Effect of free Ca^{2+} concentration on NAM superprecipitation response. Superprecipitation was started by the addition of ATP (0.1 mM in final concentration, arrow) to NAM isolated from left ventricle (A) and atrium (B).

trations (Fig. 8B). These response, which were confirmed in 4 samples, were very similar to those of scallop NAM.¹³

DISCUSSION

Atrial NAM was more sensitive to low Ca^{2+} than was ventricular NAM. The difference might be attributable to the regulatory proteins, since the reconstituted actomyosin containing atrial TM-TN complex produced higher Ca^{2+} sensitivity than did that containing ventricular TM-TN complex.

England¹⁴ reported that phosphorylation of cardiac TN-I with cyclic AMP-dependent protein kinase increases the Ca^{2+} concentration required for NAM ATPase activation, i.e., the pCa-ATPase activity curve shifts to the right. However, in the present study, the pCa required for 50% activation of atrial NAM ATPase shifted in the opposite direction.

Furthermore, MLC 2 is also phosphorylated by calmodulin-dependent protein kinase, and its phosphorylation may augment ATPase activity.^{15,16} The difference in pCa required for 50% activation in atrium and ventricle is perhaps dependent on the degree of phosphorylation of these contractile or regulatory proteins.¹⁷ The amount of phosphate covalently bound to NAM appeared greater in atrium NAM than in ventricle, although this was not statistically significant.

These results suggest that the difference in Ca^{2+} sensitivity does not involve phosphorylation of contractile proteins.

Our results excluded the possibility that the difference in pCa necessary for 50% activation was secondary to the proteolysis of TN, because a reduction in the amount of TN would increase the basal level of ATPase at low Ca^{2+} concentrations without changing the pCa required for 50% activation. Cathepsins that might have leaked from lysosomes during the NAM preparation procedure could not have been activated, because cathepsins are acid proteases and do not show any proteolysis at pH 7.¹⁸ Calcium-activated neutral proteases were also inhibited, because all procedures were performed without the addition of the Ca^{2+} or thiol reagent, which would be required for such activation,¹⁹ and the protease inhibitor, leupeptin²⁰⁻²² was always added to the extraction procedures.

The difference between atrium and ventricle in the pCa required for 50% activation suggests that there are qualitative differences among regulatory proteins, TM and/or TN. This speculation is supported by the results of SDS-PAGE (Fig. 4 and 5). The TN isolated from bovine ventricle consists of three subunits, TN-T, TN-I, and TN-C, of molecular weights 41,000, 28,000 and 18,000 respectively.³⁻⁵ The protein bands of atrial NAM indicated slightly different molecular weights, of 16,000 to 30,000. These

differences in molecular weight may reflect differences in the nature of atrial and ventricular TN. Complete isolation of TN was much more difficult from atrial than from ventricular muscle, but the procedure is now proceeding in our laboratory.

Recently, Nadal-Ginard et al²³ demonstrated that TN-T genes generate up to 64 different proteins. Tabacman et al²⁴ reported that the N-terminal region of TN-T modulates the effect of the Ca^{2+} concentration on the thin filament. According to this observation, the difference in the molecular weight of the TM-TN complexes might be small, and it is possible that the difference in the pCa necessary for 50% activation is affected by the variation in the N-terminal region of TN-T.

Kitazawa²⁵ has shown that Hill's coefficient for the development of glycerinated ventricular muscle tension is equal to one. This finding corroborates the fact that one molecule of TN-C binds to one molecule of Ca^{2+} . Hill's coefficient was similar in atrial and ventricular muscle, suggesting that one atrial TN-C molecule binds to one calcium ion.

Under high ionic strength or high MgATP concentrations, NAM dissociates into thin and thick filaments.²⁶ Atrial NAM ATPase activity was significantly higher than that of ventricle under high ionic strength or high MgATP concentrations (Fig. 6 and 7). These results indicate that atrial myosin has a greater affinity for actin than does ventricular myosin, and this was confirmed in the superprecipitation response (Fig. 8A and B). Barany et al²⁷ stressed that maximum contraction velocity correlated with Ca^{2+} -activated myosin ATPase activity, and Urthaler et al²⁸ reported that the maximum shortening velocity of atrial trabeculae is three-to fourfold higher than that of ventricular trabeculae. These findings may correspond to the stepwise change in the superprecipitation response, because the increase in turbidity during superprecipitation suggests an association between myosin and thin filaments.²⁶ The stepwise response of atrial NAM is very similar to that of scallop NAM, in which there is no TM-TN system and the regulatory system is myosin-linked.²⁹

In our laboratory *in vivo* ³¹P-NMR spectroscopy had been developed for noninvasive assessment of high-energy phosphate metabolism in the heart.^{30,31} However, even with the use of this modality it is difficult to detect higher NAM ATPase activity in atrial muscle because the

spatial resolution is not sufficient to focus exclusively on the atrium and because the atrial muscle volume is much less than that of the left ventricle.

In conclusion, our data demonstrate that there is a qualitative difference in the regulatory mechanisms of the atrium and the ventricle and constitute the first description of the possible presence of a different regulatory mechanism in the atrium.

Acknowledgments

The authors wish to thank Prof. T. Sugimoto, the Second Department of Internal Medicine, University of Tokyo, for his advice and encouragement and Misses K. Minakawa and H. Shimada for their technical and secretarial assistance.

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