A CIRCULAR DICHROISM AND BIOLOGICAL ACTIVITY STUDY ON THE HYBRID SPECIES FORMED FROM BOVINE CARDIAC AND RABBIT SKELETAL TROPONIN SUBUNITS

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Received 1 September 1977

1. Introduction

In recent years it has become apparent that physiological differences in the action of skeletal and cardiac muscle might be explained by differences in the properties and interactions of their regulatory proteins [1]. The regulation of contraction, although qualitatively similar in each muscle type, may be sufficiently different to allow the particular muscle to perform its specific function. In this study, hybrid combinations of the components of the regulatory systems of bovine cardiac and rabbit skeletal muscles were examined by circular dichroism and biological activity techniques, in order to shed some light on the specificity of intertissue troponin interactions and their possible role in explaining physiological regulatory differences between the two muscle types. The results indicate interprotein interactions among the hybrid combinations studied, as well as a potentiation of the Ca^{2+}-induced conformational change when cardiac TN-C is present, whereas no such potentiation could be discerned with skeletal TN-C. Biological activity studies employing synthetic actomyosin ATPase assay systems in the presence of tropomyosin revealed each TN-I to be more potent an inhibitor in its own parent system. The data also revealed that functional hybrids could be prepared from the various troponin subunits.

Abbreviations: CD, circular dichroism; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid; TN-C, calcium binding subunit of troponin; TN-I, inhibitory subunit; TN-T, tropomyosin binding subunit; C_sT_C, protein complex of skeletal TN-C and cardiac TN-T (other complexes are designated in the same manner); $\Delta\theta$, change in ellipticity; SAM, synthetic actomyosin; DTT, dithiothreitol; SDS, sodium dodecylsulphate

2. Materials and methods

2.1. *Protein preparations*

The procedures of Burtnick et al. [2–4] were used to isolate the troponin subunits from beef cardiac muscle. The rabbit skeletal muscle troponins were prepared by the methods developed in this laboratory [5–7]. Tropomyosin from beef cardiac and rabbit skeletal sources were prepared by the methodology of McCubbin et al. [8] with the inclusion of 0.5 mM DTT in all solutions. Protein purity was verified by SDS–polyacrylamide gel electrophoresis [9].

Cardiac and skeletal actin were prepared by the method of Spudich and Watt [10]. Myosin from beef cardiac muscle and rabbit skeletal muscle was prepared according to the procedures of Tonomura et al. [11].

2.2. *Protein concentrations*

Protein concentrations were determined spectrophotometrically on a Cary 118C instrument employing previously established extinction coefficients for the cardiac [12] and skeletal [13] troponins and tropomyosins.

2.3. *Circular dichroism*

CD measurements were performed with a Cary 6001 CD attachment to a Cary model 60 recording spectropolarimeter as described by Oikawa et al. [14]. Proteins were dialysed overnight at 4°C in a solution of 0.5 M NaCl, 50 mM Tris–HCl, pH 7.5 and 1 mM EGTA, the standard solvent system used in this work. Clarification of the protein solutions was done by centrifugation (Beckman model L ultracentrifuge) or millipore filtration. In order to obtain the Ca^{2+}-induced conformational change in ellipticity, the level of free Ca^{2+}-ions in solution was adjusted to 2 mM by means of an EGTA-containing buffer. Such a system refers to the 'plus Ca^{2+}' state, while a 'minus Ca^{2+}' state is the standard solvent system without Ca^{2+}.

Theoretical CD spectra for the complexes were calculated as before, by summing the fractional molar contributions of the uncomplexed proteins, generating a spectrum which assumed no protein interactions in the mixture [12].

2.4. *Bioassay*

Relative ATPase activities of various combinations of troponin subunits (as described in the text) plus tropomyosin with skeletal or cardiac SAM were measured by following inorganic phosphate liberation, as carried out by Burtnick et al. [3].

3. Results

3.1. *Circular dichroism*

The maximum calcium induced change in ellipticity ($\Delta\theta^{Ca^{2+}}$) occurred at 220 nm and this wavelength was, therefore, monitored for comparison purposes.

Comparison of the observed $\theta_{220\ nm}$ values with the theoretical results in the absence of Ca^{2+} (tables 1, 2), revealed that interaction occurred for most of the C–T, C–I and C–I–T complexes, according to the criterion of Burtnick and Kay [12], whereby interaction for a specific complex was indicated when the calculated and observed $[\theta]_{220\ nm}$ values differed by more than ±500°, which is considered experimental error. The only exceptions occur at the level of the C_cI_c, C_cT_c and C_sT_c complexes, where the difference values, given in the final column of both tables, are borderline in terms of significance. However, both the C_cI_c and C_cT_c complexes have been shown to form by migration patterns of samples on polyacrylamide gels in the absence of sodium dodecyl sulfate [3,4].

Representative spectra for the C_sI_c complex are presented in fig.1 where part A reveals the theoretical and observed patterns for the minus Ca^{2+} state, and part B, the corresponding spectra for the plus Ca^{2+} state. The theoretical spectra have been calculated

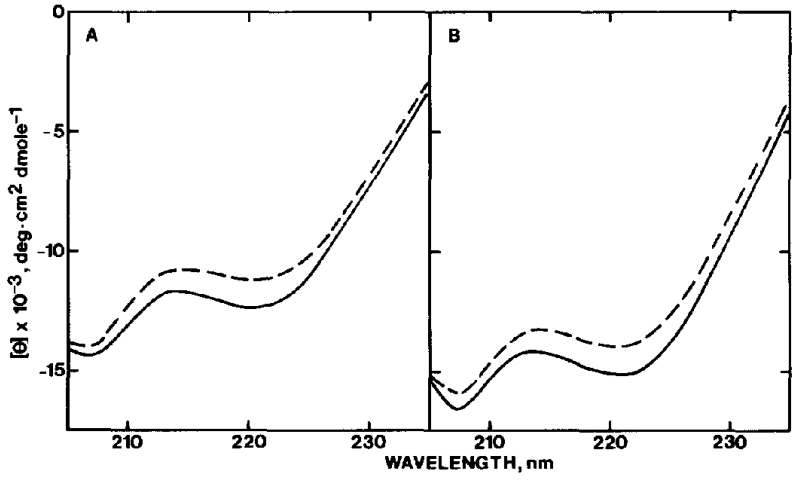

Fig.1. Theoretical (- - -) and observed (——) CD spectra for the hybrid complex skeletal TN-C and cardiac TN-I. (A) Minus Ca^{2+} state (1 mM EGTA). (B) Plus Ca^{2+} state (2 mM $CaCl_2$).

Table 1
Binary complexes

	Observed			Calculated (theoretical)			
	$[\theta]_{220\ nm}$	$\Delta\theta^{Ca^{2+}}_{220\ nm}$	$\Delta\theta^{Ca^{2+}}_{220\ nm}$%	$[\theta]_{220\ nm}$	$\Delta\theta^{Ca^{2+}}_{220\ nm}$	$\Delta\theta^{Ca^{2+}}_{220\ nm}$%	$[\theta]^{obs}_{220\ nm}-[\theta]^{calc}_{220\ nm}$
C_cI_c	−11 450			−10 950			−500
$+Ca^{2+}$	−12 750	−1300	11.4	−12 300	−1350	12.3	−450
C_cI_s	−14 046			−12 914			−1132
$+Ca^{2+}$	−16 104	−2058	14.6	−14 348	−1434	11.1	−1756
C_sI_c	−12 327			−11 174			−1153
$+Ca^{2+}$	−15 050	−2723	22.1	−13 936	−2762	24.7	−1114
C_sI_s	−18 784			−15 677			−3107
$+Ca^{2+}$	−20 235	−1451	7.7	−18 405	−2728	17.4	−1830
C_cT_c	−12 150			−12 900			+750
$+Ca^{2+}$	−12 950	−800	6.6	−13 500	−600	4.6	+550
C_cT_s	−13 515			−11 847			−1668
$+Ca^{2+}$	−15 061	−1546	11.4	−12 849	−1002	8.4	−2212
C_sT_c	−14 128			−13 503			−625
$+Ca^{2+}$	−15 454	−1326	9.4	−15 026	−1523	11.3	−428
C_sT_s	−12 019			−12 797			+778
$+Ca^{2+}$	−13 535	−1516	12.6	−14 483	−1686	13.2	+948

from those of the individual troponin subunits, assuming no interprotein interactions. Therefore, the difference between the theoretical spectra in the presence and absence of Ca^{2+} is due solely to the $\Delta\theta$ induced in TN-C by Ca^{2+}. These values were then compared with the extent of the experimentally observed Ca^{2+} induced conformational change for the corresponding complexes.

Data collected in this fashion for the C–I and C–T complexes is presented in table 1. The C_cI_c complex shows an almost identical change in ellipticity at 220 nm in the observed and theoretical spectra upon Ca^{2+} addition (−1300° versus −1350°). This suggests that the Ca^{2+}-induced conformational change occurs almost entirely at the level of TN-C, even when it is complexed with TN-I.

A different phenomenon is observed for the C_sI_s complex, where the observed $\Delta\theta^{Ca^{2+}}_{220\ nm}$ value is −1451°, as opposed to a calculated difference of −2728°. This finding implies that skeletal TN-I has an inhibitory effect upon the magnitude of the Ca^{2+}-induced change in the conformation of skeletal TN-C. On the other hand, skeletal TN-I has a potentiating effect upon the $\Delta\theta^{Ca^{2+}}_{220\ nm}$ of cardiac TN-C ($\Delta\theta^{Ca^{2+}}_{220\ nm}$ values of −2058° versus −1434° for observed and calculated spectra, respectively), while cardiac TN-I does not appear to affect the magnitude of this change in skeletal TN-C (approx. −2700° in both cases).

These effects are not carried over very dramatically to the CT complexes, where only a small potentiating effect is observed for those complexes involving cardiac TN-C. The C_cT_c complex has a larger Ca^{2+} change (−800°) compared to the theoretical spectrum, and the same trend is also observed for the C_cT_s complex (−1546° versus −1002°). In contrast, in the case of the complexes involving skeletal TN-C, the observed change is slightly less than the theoretical one.

Examination of the data for the ternary complexes (table 2) reveals several important features at the level of the TN-C component. Those complexes containing skeletal TN-C do not show any potentiation, when the $\Delta\theta^{Ca^{2+}}_{220\ nm}$ for the observed and theoretical spectra are compared. Although large changes in $\theta_{220\ nm}$ are noted upon Ca^{2+} addition (approx. −1500°), they are predictable

Table 2
Ternary complexes

	Observed			Calculated (theoretical)			
	$[\theta]_{220\ nm}$	$\Delta\theta^{Ca^{2+}}_{220\ nm}$	$\Delta\theta^{Ca^{2+}}_{220\ nm}$%	$[\theta]_{220\ nm}$	$\Delta\theta^{Ca^{2+}}_{220\ nm}$	$\Delta\theta^{Ca^{2+}}_{220\ nm}$%	$[\theta]^{obs}_{220\ nm}-[\theta]^{calc}_{220\ nm}$
$C_sI_sT_s$	−14 556			−11 442			−3114
$+Ca^{2+}$	−15 924	−1368	9.4	−12 783	−1341	11.7	−3141
$C_sI_sT_c$	−15 940			−13 415			−2525
$+Ca^{2+}$	−17 413	−1473	9.2	−14 512	−1097	8.2	−2901
$C_sI_cT_s$	−11 200			−9707			−1493
$+Ca^{2+}$	−12 433	−1233	11.0	−11 117	−1410	14.5	−1316
$C_cI_sT_s$	−11 274			−10 354			−920
$+Ca^{2+}$	−12 880	−1606	14.2	−11 039	−685	6.6	−1841
$C_sI_cT_c$	−12 492			−11 225			−1267
$+Ca^{2+}$	−13 973	−1481	11.8	−12 529	−1304	11.6	−1444
$C_cI_cT_s$	−10 996			−9924			−1072
$+Ca^{2+}$	−12 003	−1007	9.2	−10 987	−1063	10.7	−1016
$C_cI_sT_c$	−15 968			−12 656			−3312
$+Ca^{2+}$	−17 820	−1852	11.6	−13 349	−693	5.5	−4471
$C_cI_cT_c$	−12 200			−11 500			−700
$+Ca^{2+}$	−13 430	−1230	10.1	−12 200	−700	6.1	−1230

and suggest that the reconstituted troponin is interacting with calcium in a similar manner to the uncomplexed TN-C. In contrast, those complexes containing cardiac TN-C all show large potentiation effects, a phenomenon originally observed by Burtnick and Kay for the exclusively cardiac ternary complex [12]. Typically, the observed $\Delta\theta^{Ca^{2+}}_{220\ nm}$ is about double that predicted by the theoretical calculations. For example, with the $C_cI_sT_c$ ternary complex, the observed $\Delta\theta^{Ca^{2+}}_{220\ nm}$ is −1852° as opposed to a calculated value of −693°.

3.2. *Bioassay*

As shown in figs 2 and 3, both skeletal and cardiac TN-I have inhibitory activity in the skeletal and cardiac SAM systems. An interesting feature is that while each TN-I component is operative in both SAM systems, a given TN-I is most active with its 'parent' actomyosin. Syska et al. [15] and Tsukui and Ebashi [16] also found that cardiac TN-I was inhibitory in a desensitized skeletal actomyosin system. The present results confirm that observation, but extend it to comparative studies with both the skeletal and cardiac actomyosin systems, and thus represent a more complete and relevant picture.

It is also noteworthy that cardiac TN-I is a less effective inhibitor against cardiac SAM, than is skeletal TN-I versus its parent actomyosin system. In this connection, to produce 50% inhibition in the

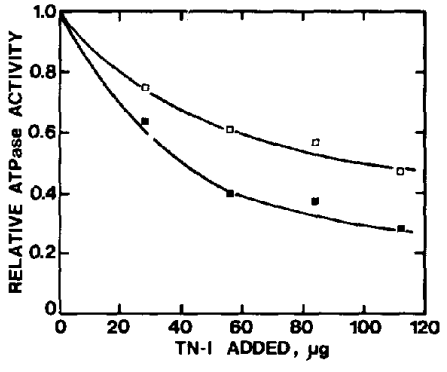

Fig.2. Relative ATPase activity of cardiac SAM as a function of amount of TN-I added to the assay system for cardiac TN-I (■–■) and skeletal TN-I (□–□).

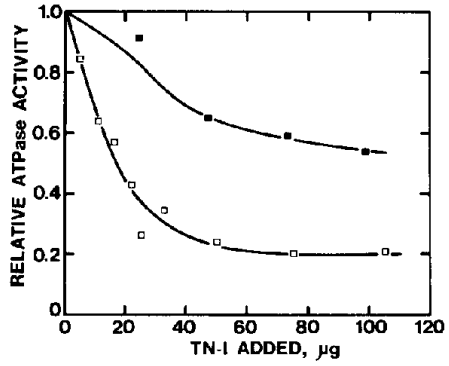

Fig.3. Relative ATPase activity of skeletal SAM as a function of amount of TN-I added to the assay system for cardiac TN-I (■—■) and skeletal TN-I (□—□).

cardiac SAM system requires 50 μg cardiac TN-I, whereas only 20 μg skeletal TN-I will produce the same degree of inhibition in the skeletal actomyosin system. This finding of a smaller degree of inhibition of cardiac actomyosin ATPase activity by cardiac TN-I may have physiological relevance, since cardiac muscle must function continuously, without an opportunity for rest and recovery.

Addition of the TN-C components reversed the inhibitory effects of the TN-I subunits (table 3)

Table 3
Restoration of ATPase activity by TN-C to skeletal SAM system partially inhibited by TN-I

	Skeletal TN-I	Cardiac TN-I	Skeletal TN-C	Cardiac TN-C	Relative activity
1.	–	–	–	–	1.0
2.	25 μg	–	0	–	0.27
	25 μg	–	15 μg	–	0.73
	25 μg	–	45 μg	–	0.81
	25 μg	–	75 μg	–	0.93
3.	25 μg	–	–	0	0.27
	25 μg	–	–	15 μg	0.73
	25 μg	–	–	45 μg	0.76
	25 μg	–	–	75 μg	0.86
4.	–	71 μg	0	–	0.60
	–	71 μg	45 μg	–	0.81
	–	71 μg	75 μg	–	0.90
	–	71 μg	112 μg	–	0.98
5.	–	71 μg	–	0	0.60
	–	71 μg	–	45 μg	0.78
	–	71 μg	–	75 μg	0.79
	–	71 μg	–	112 μg	0.81

Note: The control contained 750 μg synthetic actomyosin and 50 μg tropomyosin in 10 mM Tris–HCl, pH 7.6, 2.5 mM $MgCl_2$, 1 mM EGTA and 2.5 mM ATP at 20°C

Table 4
Ca^{2+} activation of ATPase activity of SAM (A-skeletal, B-cardiac) with reconstituted hybrid troponin

	Skeletal troponins			Cardiac troponins			$CaCl_2$	Relative activity
	TN-C	TN-I	TN-T	TN-C	TN-I	TN-T	(2 mM)	
A.	30 μg	25 μg	40 μg					0.69
	30 μg	25 μg	40 μg					0.93
				75 μg	71 μg	120 μg		0.74
				75 μg	71 μg	120 μg		1.0
	30 μg	25 μg	–			40 μg		0.70
	30 μg	25 μg	–			40 μg		0.98
B.				68 μg	56 μg	84 μg		0.47
				68 μg	56 μg	84 μg		0.69
	62 μg	56 μg	75 μg					0.53
	62 μg	56 μg	75 μg					0.77
			75 μg	68 μg	56 μg	–		0.60
			75 μg	68 μg	56 μg	–		0.76
	62 μg	56 μg	–			84 μg		0.32
	62 μg	56 μg	–			84 μg		0.41

while incorporation of TN-T into the assay systems with TN-I and TN-C conferred Ca^{2+} sensitivity on the regulatory process (table 4). In the presence of EGTA, the ATPase activities were inhibited, whereas in the presence of Ca^{2+} reversal of these inhibitions was noted.

4. Conclusion

The biological activity of the hybrid complexes studied is in good agreement with the circular dichroism data, which indicates that interaction occurs among the troponin subunits of the two tissues. This implies that the regulatory proteins in each system are structurally similar enough to form functional hybrid complexes. However, there are known differences between the same proteins within the two tissues, reflected largely at the level of primary sequence and molecular weight [2–4], number of calcium binding sites [17–19] and phosphorylation characteristics in vitro and in vivo [20], which results in very specific interactions within each system. These differences show up in the CD and biological properties of the hybrid complexes and suggest that although both the skeletal and cardiac regulatory systems function in a qualitatively similar manner, they are nonetheless unique in several aspects.

Both the potentiation of the Ca^{2+}-induced conformational change in those ternary complexes containing cardiac TN-C, and the smaller degree of inhibition elicited by cardiac TN-I in its parent actomyosin system, are expressions of the unique differences between the two muscle regulatory systems, which may well have physiological relevance.

Acknowledgements

The authors are indebted to K. Oikawa, A. Keri and V. Ledsham for their excellent technical assistance. They also thank Dr L. D. Burtnick for helpful discussions. Special thanks are also due to Mr V. Jones of Swift & Co. for the generous supply of fresh beef hearts. M. T. Hincke is grateful to the National Research Council of Canada for receipt of a postgraduate scholarship.

References

[1] Ebashi, S., Masaki, T. and Tsukui, R. (1974) Adv. Cardiol. 12, 59–69.
[2] Burtnick, L. D., McCubbin, W. D. and Kay, C. M. (1975) Can. J. Biochem. 53, 15–20.
[3] Burtnick, L. D., McCubbin, W. D. and Kay, C. M. (1975) Can. J. Biochem. 53, 1207–1213.
[4] Burtnick, L. D., McCubbin, W. D. and Kay, C. M. (1976) Can. J. Biochem. 53, 546–552.
[5] McCubbin, W. D. and Kay, C. M. (1973) Biochemistry 12, 4232.
[6] Mani, R. S., McCubbin, W. D. and Kay, C. M. (1973) FEBS Lett. 29, 243.
[7] Mani, R. S., McCubbin, W. D. and Kay, C. M. (1974) FEBS Lett. 38, 357.
[8] McCubbin, W. D., Konba, R. F. and Kay, C. M. (1967) Biochemistry 6, 2417–2425.
[9] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[10] Spudich, J. A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
[11] Tonomura, Y., Appel, P. and Morabs, M. T. (1966) Biochemistry 5, 515–521.
[12] Burtnick, L. D. and Kay, C. M. (1976) FEBS Lett. 65, 234–237.
[13] Mani, R. S., McCubbin, W. D. and Kay, C. M. (1975) FEBS Lett. 52, 127–131.
[14] Oikawa, K., Kay, C. M. and McCubbin, W. D. (1968) Biochim. Biophys. Acta 168, 164–167.
[15] Syska, H., Perry, S. V. and Trayer, I. P. (1974) FEBS Lett. 40, 253–257.
[16] Tsukui, R. and Ebashi, S. (1973) J. Biochem. (Tokyo) 73, 1119–1121.
[17] Van Eerd, J. P. and Takahashi, K. (1975) Biochem. Biophys. Res. Commun. 64, 122–127.
[18] Buss, J. E. and Stull, J. T. (1977) FEBS Lett. 73, 101–104.
[19] Burtnick, L. D. and Kay, C. M. (1977) FEBS Lett. 75, 105–110.
[20] Stull, J. T. and Buss, J. E. (1977) J. Biol. Chem. 252, 851–857.