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DUAL ROLE OF CALSEQUESTRIN AS SUBSTRATE AND INHIBITOR OF CASEIN KINASE-1 AND CASEIN KINASE-2

Sergio Salvatori[*,#], Sandra Furlan[*] and Flavio Meggio[**]

[*] *CNR Centro di Studio per la Biologia e la Fisiopatologia Muscolare c/o Dipartimento di Scienze Biomediche Sperimentali and* [**] *CNR Centro per lo Studio della Fisiologia dei Mitocondri c/o Dipartimento di Chimica Biologica, Universita' di Padova, via Trieste 75, 35121-Padova, ITALY*

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Calsequestrin from different muscle tissues and species has been phosphorylated by casein kinase-1 and casein kinase-2, in the conditions previously reported by Cala and Jones (J. Biol. Chem. **266**, 391-398, 1991). Results indicates that rabbit cardiac and skeletal calsequestrin and frog skeletal calsequestrin are phosphorylated by both casein kinase-1 and casein kinase-2, at variance with chicken skeletal calsequestrin which is a poor substrate for both enzymes. We also observed that chicken calsequestrin is able to inhibit phosphorylation of cardiac calsequestrin, as well as other specific substrates, when added together to the assay medium.

Calsequestrin (CS) is the major intralumenal calcium binding protein of the sarcoplasmic reticulum (SR). Since the first reports on CS (1, 2) the main function has been indicated in buffering calcium, due to its high binding capacity. Many isoforms of CS have been described and sequenced (3-8). Recently, it has been shown that cardiac and skeletal CS are phosphorylated by casein kinase-2 (CK2) (9, 10), a ubiquitous Ser/Thr-specific protein kinase supposed to play a central role in cell regulation and preferring acidic protein substrates (11, 12). In particular, cardiac CS seems to be the best substrate for CK2 due to the presence of a cluster of three closely spaced serine residues included in the polyaspartyl COOH-terminal tail of the molecule. Also rabbit skeletal CS which lacks the polyaspartate tail (5,6) undergoes phosphorylation by CK2, although at lower extent (9). Chicken and frog skeletal isoforms of CS have an uninterrupted tail of polyaspartate (7, 8), thus lacking the predominant phosphorylation sites of cardiac CS. The present work shows that rabbit cardiac and skeletal CSs and frog skeletal CS are *in vitro* substrates not only for CK2 but also for casein kinase-1 (CK1), another ubiquitous acidophylic Ser/Thr protein kinase

To whom any correspondence should be addressed. FAX: +.39.49.8286576 / e-mail: patgen08@cribi1.bio.unipd.it.

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biochemically and immunologically distinct from CK2 (11, 12) and that chicken CS has an inhibitory effect on the activity of both CK1 and CK2.

Materials and Methods

Chemicals. [γ-^{32}P]ATP was purchased from DuPont-New England Nuclear. All other chemicals were analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO), BDH (Poole, England), Merck A.G. (Darmstadt, Germany). Synthetic peptides RRRAADSDDDDD and an octadecapeptide reproducing with slight modifications the site 174 of inhibitor-2 of protein phosphatase-1 (13) were kindly provided by Dr. O. Marin (Padova)
Calsequestrin purification. CS was isolated from cardiac and skeletal muscles of rabbit, and from skeletal muscles of chicken and frog, by ammonium sulphate fractionation and chromatography on DEAE-cellulose and Phenyl-sepharose (Pharmacia, Uppsala, Sweden) as described by Slupski *et al.* (14).
Casein kinases purification. CK1 and CK2 were purified as described by Meggio et al. (15). The specific activity was determined to be 35 and 28 U/mg for CK2 and CK1 respectively, one unit being the amount of enzyme transferring 1 nmol of P/min to casein.
Phosphorylation assay. Phosphorylation of calsequestrins was carried out according to Cala and Jones (9) with slight modifications as follows: assays were carried out in a final volume of 25 μl in 20 mM MOPS pH 7.4, 6 mM EGTA, 10 mM $MgCl_2$, 0.15 M NaCl, 0.2% Triton X100. In CK1 phosphorylation assay 10 μg/ml heparin was added to prevent possible phosphorylation due to contaminating CK2. Reaction was initiated by addition of 50 μM ATP (10^9 dpm), carried out as indicated in the figure legends and stopped by adding SDS-solubilization buffer (16). Incorporation of ^{32}P-phosphate into proteins was checked by autoradiography and measured after excision of labelled bands from the essiccated electrophoretic gel slab. Peptides phosphorylation was accomplished essentially at the same conditions described above and evaluated as in (17).
Electrophoresis. SDS-PAGE was carried out according to Laemmli (16) using 7.5% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue.
Determination of protein concentration. Protein concentration was determined according to Lowry *et al.* (18) using bovine serum albumine as standard.
Kinetic constants. Kinetic constants of protein and peptide substrates were determined by regression analysis of double-reciprocal plots constructed from initial rate measurements.

Results and Discussion

a) *CS as a substrate of casein kinases -1 and -2.* Fig. 1 shows autoradiograms obtained at increasing incubation time of rabbit cardiac and skeletal CS and chicken and frog skeletal CS with CK1 (panel a) and CK2 (panel b). It is evident from P_i incorporation data reported in Fig. 1 that: 1) cardiac CS appears to be an excellent substrate for CK2, as expected, but it is strongly phosphorylated also by CK1, phosphoserine being the only radiolabeled phosphoaminoacid detected in both cases (not shown); 2) chicken skeletal CS is the poorest substrate for both phosphorylating enzymes; 3) rabbit and frog skeletal CS show an intermediate phosphorylation by CK1 and a phosphorylation level by CK2 comparable to that of cardiac isoform .

Table I shows kinetic parameters for casein kinases substrates. The highest Vmax value was obtained for cardiac CS phosphorylated by CK2, the lowest being the chicken skeletal muscle

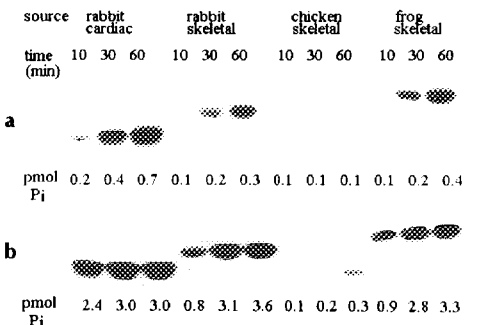

Fig. 1. Time course of phosphorylation of purified rabbit cardiac and skeletal calsequestrins, and chicken and frog skeletal calsequestrins by CK1 and CK2. Purified calsequestrins (2 μM) were phosphorylated (see Materials and Methods section) in the presence of 6.4 μg/ml CK1 (panel a) or 12 μg/ml CK2 (panel b), for 10, 30 and 60 minutes, as indicated. Samples were analyzed by SDS-PAGE. The stained and essiccated gel was exposed to an X-ray film at -80°C for 24 h (panel a) and 14 h (panel b). Protein bands were excised and radioactivity was quantified by liquid scintillation counting. Autoradiography is shown together with pmoles of Pi incorporated, for each lane.

isoform phosphorylated by CK1. The absolute values obtained are quite different from those previously reported for CK2 by Cala and Jones (9) and the phosphatase pretreatment was ineffective in increasing the maximal phosphorylation (data not shown). It should be underlined,

TABLE I

Kinetic parameters for casein kinases substrates

Substrate	Enzyme	Km (μM)	Vmax (nmol/min/mg CK)	Vmax/Km
rabbit cardiac CS	CK1	1.8	3.0	1.6
	CK2	4.4	10.2	2.3
rabbit skeletal CS	CK1	0.75	0.9	1.2
	CK2	2.7	0.4	1.5
chicken skeletal CS	CK1	0.02	0.06	3.0
	CK2	0.5	0.08	0.2
frog skeletal CS	CK1	0.13	0.09	0.69
	CK2	0.55	0.81	1.5

Purified calsequestrins, from 1 to 10 μM, were phosphorylated for 10 minutes in the presence of either 6.4 μg/ml of CK1 or 12 μg/ml of CK2 and 50 μM ATP. Calsequestrins phosphorylation was analyzed by autoradiography after SDS-PAGE and radioactivity content of protein bands measured by scintillation counting.

however, that we found a substrate inhibition effect for all CSs investigated , at a protein concentration higher than 3 μM, wich could therefore alters the current evaluation. Km values calculated on the first portion of the kinetics (below 3 μM) resulted in the range of 1 to 10 μM.

b) *CS as an inhibitor of casein kinases -1 and -2* . The low level of phosphorylation of chicken CS is not easily explained by the absence of serine residues in the very acidic tail of polyaspartate, since this tail is virtually lacking in the rabbit skeletal CS (5, 6) were phosphothreonine was the main phosphorylatable residue (9). One possibility is that the polyanionic tail of chicken CS could act as a powerful inhibitor of casein kinases, polyaspartic and polyglutamic acid being strong inhibitors of CK2 (19). Since it is known that also frog CS possesses an uninterrupted polyaspartate tail (8) we performed the experiment illustrated in Fig. 2, where the phosphorylation of cardiac CS was carried out in the presence of the other CSs. We found a strong inhibitory effect (about 85%) of chicken CS on the cardiac CS phosphorylation (Table II) for both type of phosphorylating enzymes. The same inhibition was also detected toward specific peptide substrates of CK2 and CK1 (data not shown). On the other hand, while frog skeletal CS appears to be almost ineffective with both kinases, the rabbit skeletal CS shows a significant inhibitory effect on CK1 but not on CK2, suggesting a different type of inhibition. Clearly, the only presence of the polyaspartate tail, which in frog is longer than in chicken CS (8), is not adequate to explain the inhibitory effect. Preliminary results indicate that frog CS is mainly phosphorylated at threonine residues, possibly the same which is phosphorylated in skeletal CS, while chicken CS is very slowly phosphorylated at a seryl residue.

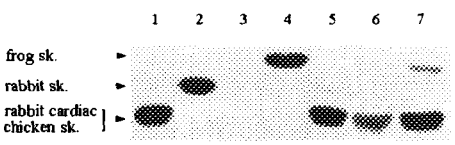

Fig. 2. Inhibitory effect of chicken skeletal muscle calsequestrin on rabbit cardiac calsequestrin phosphorylation by CK2. Phosphorylation assay was carried out as in Fig. 1 with the following changes: incubation time, 10 min; cardiac CS concentration, 1 μM; other CSs concentration, 2 μM. After SDS-PAGE analysis, the gel was stained with Coomassie Brilliant Blue and essiccated. The autoradiography is shown. Stained bands were excised and radioactivity was measured by scintillation counting.
Key to lanes: 1) cardiac CS; 2) rabbit skeletal CS; 3) chicken skeletal CS; 4) frog skeletal CS; 5) rabbit cardiac CS and rabbit skeletal CS; 6) rabbit cardiac CS and chicken skeletal CS; 7) rabbit cardiac CS and frog skeletal CS.

TABLE II

Inhibitory effect of chicken calsequestrin on cardiac calsequestrin phosphorylated by casein kinases -1 and -2

Added CS	[CS] μM	% inhibition with CK1	CK2
rabbit skeletal	2	17	9
	3	63	0
frog skeletal	2	2	2
	3	5	8
chicken skeletal	2	56	57
	3	87	80

Rabbit cardiac calsequestrin (1 μM) was used as substrate for the phosphorylation assay, carried out in the same conditions reported in the legend of Tab I, both alone and in the presence of each of the other calsequestrins (2 μM or 3 μM). Inhibition is reported as a percentage, calculated assuming the phosphorylation level of rabbit cardiac CS alone, as 100%.

Based on the following partial sequence comparison:

E^{344} D V L E G E I N T E D D D D^{358}	rabbit skeletal CS (5)
E D V L S G K I N T E D D D D	chicken skeletal CS (7)
E D V L S G K I N T E D D D N	dog cardiac CS (6)
E D V L E G E V N T E D D D D	frog skeletal CS (8)

we suggest that the presence of Lys^{350} at position -3 from Thr^{353} in chicken CS may be the explanation for both its low level of phosphorylation and, perhaps, for the inhibitory effect, at least on CK2. N-terminally located basic residues, in fact, have been found to negatively affect the site recognition by CK2 (19). Moreover, frog CS which is phosphorylated at a threonyl residue, has a glutamate residue at position 350 and does not show any inhibitory effect on casein kinases. On the other hand, cardiac CS which also possesses a Lys^{350} and is readily phosphorylated at the serine cluster in the last part of the molecule, is not phosphorylated at Thr^{352} (9).

In order to preliminarily investigate on what kind of inhibition might be involved, we performed the experiments reported in Fig. 3 using RRRAADSDDDDD and a peptide derived from site 174 of inhibitor 2 (13) as specific substrate for CK2 and CK1, respectively (F. Meggio, O. Marin and L.A. Pinna, manuscript in preparation). The Ki value of 1.4 μM can be easily determined for CK2, the inhibition of this casein kinase being of the competitive type, while a different mechanism has to be invocated in the case of CK1.

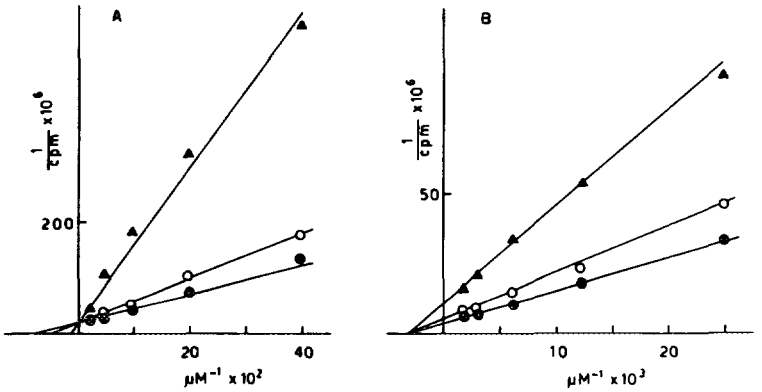

Fig. 3. Double-reciprocal plots for casein kinase-2 (panel A) and casein kinase-1 (panel B) inhibition by chicken skeletal calsequestrin. Phosphorylation conditions are described under Materials and Methods. The phosphorylatable specific peptide substrates, RRRAADSDDDDD in the case of CK2 and an octadecapeptide derived from site 174 of inhibitor-2 of protein phosphatase-1 (13) in the case of CK1, were assayed either in the absence (●) or in the presence of chicken calsequestrin (1.2 μM (○) and 4.8 μM (▲) in A and 0.48 μM (○) and 1.92 μM (▲) in B).

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