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Cellular Distribution of Sarcoplasmic Calcium-Binding Proteins by Immunofluorescence*

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Summary. Specific antibodies against carp paravalbumin, crayfish calcium binding protein and crayfish arginine kinase were used for indirect immunofluorescence localization of the respective proteins. Simultaneous staining of the same muscle sections with human serum containing anti-actin autoantibodies served as a probe to identify the isotropic band.

Parvalbumin appears to be evenly distributed in carp white muscle. The crayfish calcium binding protein however shows a distinct localization, in the isotropic band, coincident with the actin staining. Arginine kinase, which has the same molecular weight and is extractible in the same way as the calcium binding protein, does not show this distinct localization, but is evenly present in crayfish tail muscle, similarly to parvalbumin.

The possible meaning of the different distribution of the two calcium binding proteins is discussed.

Introduction

The concerted regulation of muscular contraction and glycogenolysis has been explained in vertebrates by the respective sensitivity to calcium of troponin and phosphorylase kinase (Kretsinger, 1976b; Fischer et al., 1970). Inside the muscle cell the uptake and release of calcium is under the dependence of the sarcoplasmic reticulum (Benzonana et al., 1974a) or perhaps mitochondria (Carafoli et al., 1975). Besides this generally accepted basic mechanism, the existence in vertebrate white muscle of an important calcium sink formed by parvalbumins has been overlooked by workers in the field of calcium regulation. In spite of the complete elucidation of the structure of these proteins (Collins, 1976), of intensive studies on their metal binding characteristics (Kretsinger,

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1976a) and on their physiological importance (Kretsinger, 1976b), their high concentration in many muscles has not received any explanation up to now. In search for the physiological function of soluble calcium binding proteins, we decided to look towards the invertebrates, where more simple mechanisms could be hopefully expected.

Fresh water crayfish (*Astacus Pontastacus leptodactylus*) has separated from the main evolutionary line probably 400 million years ago. The study of its muscular system enabled us to show that there is no fundamental biochemical difference from that of rabbit (Benzonana et al., 1974a). The actin-bound calcium regulatory unit is composed of tropomyosin and troponin, the latter being made up of 3 subunits, as in rabbit. Interestingly, crayfish sarcoplasm does not contain parvalbumins as vertebrates do. A different calcium binding protein of higher molecular weight (Table 1) made of 2 probably identical subunits is present (Cox et al., 1976b). This protein is able to bind essentially all the calcium found in crayfish muscle myogen (Benzonana et al., 1974b).

Both parvalbumin and crayfish calcium binding protein are easily extracted in similar very mild conditions (Cox et al., 1976a), thus they apparently belong to the myoplasm. However this does not exclude their localization in microcompartments within the muscle cell.

In order to verify this hypothesis, we studied the localization of soluble sarcoplasmic calcium binding proteins from a vertebrate (carp) and an invertebrate (fresh water crayfish) organism.

Materials and Methods

1. Proteins Used as Antigens

Parvalbumin: carp parvalbumins were prepared according to Pechère et al. (1971). Component number 3 (one of the main parvalbumins of this organism) was isolated after chromatography on DEAE cellulose and gave a single band by disc gel electrophoresis.

Arginine kinase and calcium binding protein were extracted simultaneously from crayfish tail muscle and separated from each other by DEAE cellulose chromatography (Benzonana et al., 1974b). Both proteins were pure as judged by disc gel electrophoresis. Some of the properties of parvalbumin, arginine kinase and calcium binding proteins are summarized in Table 1.

Table 1. Physical Properties of 3 Sarcoplasmic Proteins

	Carp parvalbumin component 3	Crayfish calcium-binding protein	Crayfish arginine kinase
MW native protein	11,489[a]	44,000[c]	37,000[b]
MW SDS treated	11,500[b]	22,000[c]	45,000[b]
Calcium atoms per mole of protein	2[a]	6[c]	0[b]

[a] Pechère et al., (1973)
[b] Unpublished experiments
[c] Cox et al., (1976b)

2. Antibodies

Against Actin: Serum containing specific antiactin autoantibodies was obtained from a patient suffering from chronic aggressive hepatitis (Gabbiani et al., 1973, Lidman et al., 1976). The following methods were used to ascertain that the antibody was specific against actin only and not against any other contractile or regulatory protein: pure actin (Spudich and Watt, 1971), thrombosthenin (Probst and Lüscher, 1972), tropomyosin (Hartschorne and Mueller, 1969) and troponin (Eisenberg and Kielley, 1974) were prepared and their respective purities checked by disc gel electrophoresis. They were then added at different concentrations to aliquots of the antiactin serum. Eventual precipitates were discarded after centrifugation. The antibody specificity of the serum thus treated was checked by staining rat skeletal muscle sections and Ouchterlony's immunodiffusion. No decrease in the antiactin activity was observed after incubation with tropomyosin, troponin, whereas after incubation with actin or thrombosthenin the staining of I bands of skeletal muscle disappeared. The specificity of the antiactin serum will be detailed elsewhere (Chaponnier et al., in press).

Antibodies against carp parvalbumin, crayfish arginine kinase and calcium binding protein: rabbits (2.5–3 kg) were first injected both intramuscularly and subcutaneously with 20 mg of protein dissolved in 2 ml of 0.145 M NaCl pH 7.0 emulsified with 2 ml of Freund's complete adjuvant (Difco Chemical Co). After 3 weeks, 3 additional subcutaneous injections were performed at weekly intervals with 5 mg of one of the proteins in 0.5 ml of 0.145 M NaCl emulsified with 0.5 ml of Freund's incomplete adjuvant. The rabbits were bled 10 days after the last injection via heart puncture. Immunoglobulins were isolated by salting out and ion exchange chromatography carried out as described by Harboe and Ingild (1973); their specificity was checked by the double diffusion method (Ouchterlony and Nilsson, 1974).

Fluorescein and Rhodamine Conjugated γ Globulin Fractions. The fluorescein conjugated γ globulins of goat against human γ globulin were obtained from Miles Laboratories, Lausanne, Switzerland (code No 64-170-1). Rhodamine conjugated antibodies were prepared in our laboratory by coupling a γ globulin fraction of sheep serum against rabbit γ globulin with rhodamine by the technique of Cebra and Goldstein (1965).

3. Preparation and Staining of Sections

Immediately after the sacrifice of carp or crayfish, selected muscle fibers (white lateral muscle from carp, ventral superficial from crayfish) were excised and stretched in saline buffer for 30 min. Shorter times of stretching (down to 5 min) gave no sensible differences, except that the stretching was less well maintained thereafter, and examination of the sections was more difficult. The fibers were then frozen with carbon dioxide, and cryostat sections of 4 µm prepared. They were fixed with acetone for 5 min at −20° C, dried in air at 20° C for 2 h and treated for 15 min in a moist chamber with one of the rabbit γ globulin fractions against: parvalbumin, calcium binding protein, arginine kinase, or, for control, with normal rabbit serum. They were then washed several times with phosphate buffer (50 mM pH 7.2) containing 0.15 M NaCl (PBS) and treated with a rhodamine labelled γ globulin fraction of sheep serum against rabbit γ globulin. A similar staining for the localization of actin was performed on the same sections: human antiactin serum was added to the sections and left for 15 min. After 3 washings with PBS, a fluoresceine labelled γ globulin fraction of goat serum against human γ globulin was added; 15 min later, the sections were washed again 3 times. Photographs were taken at a magnification of 400 times with a Zeiss UV photomicroscope with epi-illumination and barrier filters specific for fluoresceine and rhodamine respectively. Pictures were taken using Anscochrome (Gaf Corporation, New York) color slide film (sensitivity 500 ASA daylight). Thus on the original pictures, actin is identified by a yellow-green color and the 3 other proteins by a red one.

Results

Figure 1 is an example of the pictures obtained on a section of stretched fiber from rat muscle. On the right side, the section is seen directly by phase contrast

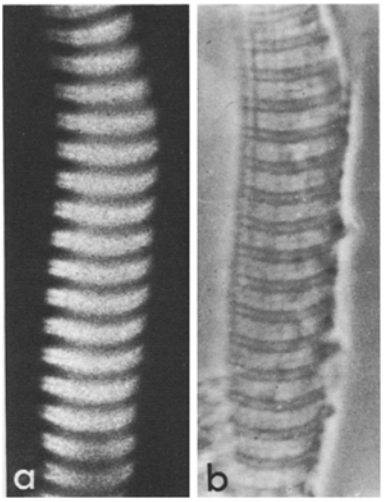

Fig. 1a and b. Immunofluorescent localization of actin in rat muscle: **a** UV illumination, **b** phase contrast. Enlargement: ×1700

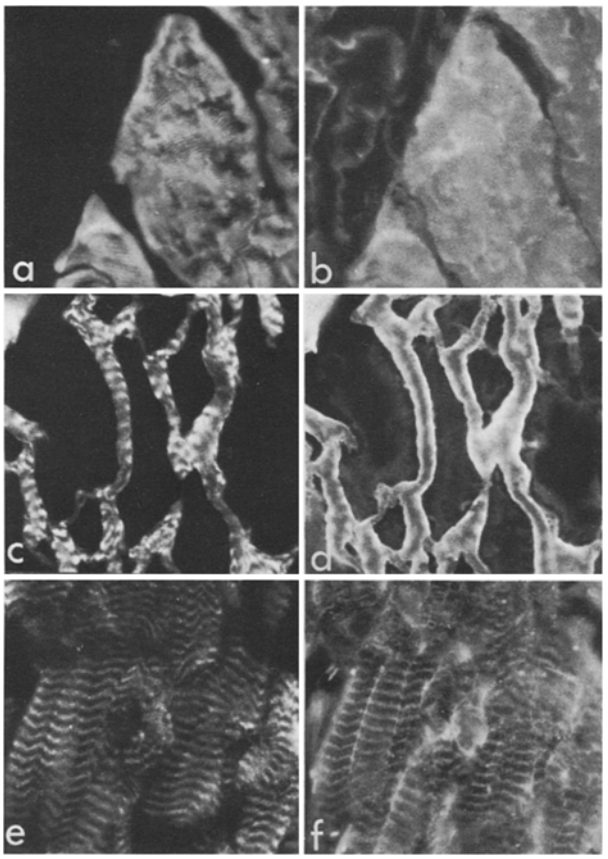

Fig. 2a–f. Immunofluorescent localization of carp parvalbumin (**b**); crayfish arginine kinase (**d**); crayfish calcium binding protein (**f**). Actin staining is shown on pictures (**a**), (**c**) and (**e**), and corresponds respectively to the preparations shown on pictures (**b**), (**d**) and (**f**). Enlargement: ×700

under visible light, while on the left side, the response of the fluorescent probe under ultraviolet illumination (fixed on actin) is shown. As can be seen, there is a perfect correspondence between the clear I bands (in direct light) and the clear fluorescent bands.

The upper part of Figure 2 shows a carp muscle section, stained for actin (a) and parvalbumin (b). Striations – corresponding to the I band – are visible in some places (a). This is not as clear as in the rat, since carp fibers are very fragile; only very slight stretching can be achieved before rupture occurs. In contrast, on Figure 2 (b) the fluorescence is evenly distributed on the whole surface of the section, suggesting either that parvalbumin is naturally present everywhere, or that during stretching it has diffused in the whole muscle.

Striations are clearly visible for actin (c) in crayfish muscle. The staining made for arginine kinase shows fluorescent fibers (d), however no band which could correlate with actin is visible. The darker line running all along the middle of the fibers in the center of (d) does not correspond to any known structure but suggests a higher concentration of enzyme at the border of the fibers. The general appearance of (d) can be compared with that of parvalbumin: with respect to the I and A band, an even distribution of the protein appears probable.

Figure 2 (f) shows the distribution of the crayfish calcium binding protein. Contrary to (b) and (d), alternating dark and bright transverse striations appear on (f). The correspondence between the bands of actin (e) and those of calcium binding protein (f) can be observed: it implies a similar distribution of the two proteins.

Discussion

The calcium binding protein from crustacean sarcoplasm shares some of the characteristic features of parvalbumins, their counterparts found in vertebrates (Pechère et al., 1973, Lehky et al., 1974). Crayfish calcium binding protein is easily extracted from muscle. A simple homogenization of crayfish muscle in a low ionic strength buffer allows the recovery of 90% of the total of this protein (Cox et al., 1976b). The same extraction procedure is valid for fish parvalbumin (Pechère et al., 1973). Thus neither type of calcium binding proteins seems to be strongly bound to an insoluble matrix.

A functional analogy between crustacean calcium binding proteins and parvalbumins of vertebrates has been postulated (Cox et al., 1976b) on the basis of the similarity of their relative concentration within different muscles with respect to tension development, contraction speed and mitochondria content. Parvalbumins as well as crustacean calcium binding proteins are abundant in fast muscles. However they do not seem to be essential for the contraction of some other muscles: e.g. they are absent or found only in minute amount in heart muscle (Gosselin-Rey, 1974, Cox et al., 1976b).

According to the present research, crayfish calcium binding protein is located at the site of the isotropic bands, while parvalbumin appears evenly distributed in carp white muscle. We do not think that the omnipresence of parvalbumin in muscle is due to its small size (Table 1), which could enable the protein

to diffuse very quickly in the whole muscle during stretching. Indeed various experiments made with different (shorter than 30 min) stretching times revealed no fundamental difference in the various sections after staining for parvalbumin, and no striations were in any way apparent. Moreover, two abundant proteins of crayfish sarcoplasm, namely calcium binding protein and arginine kinase, which have molecular weights of the same magnitude (Table 1), are extractible in the same conditions and present in similar concentrations, do not appear to be distributed in the same way in muscle. This supports the validity of the technique used here.

In spite of the various arguments for a functional analogy of parvalbumins and crustacean calcium binding protein, we have found a different distribution in muscle for these two types of calcium binding proteins in their respective organisms. This can suggest that the physiological significance of the two proteins is different, or at least that the crayfish calcium binding protein binds more tightly to a particulate structure of muscle than parvalbumin does. Indeed, distinct amino acid compositions and other molecular properties (Cox et al., 1976b), or different calcium binding characteristics (Wnuk et al., 1976), can cause a different behaviour in protein-protein interactions. A second hypothesis is that the need for calcium and hence its distribution is not the same in the muscles of carp and crayfish: different distributions of parvalbumin and calcium binding protein can reflect these dissimilarities.

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