THE DISTRIBUTION OF PARVALBUMINS IN MUSCLE AND IN OTHER TISSUES

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1. Introduction

Parvalbumins are acidic calcium-binding proteins, with a mol. wt around 12 000, present in high concentration (approx. 1 mM) in the myogen of lower vertebrate's white muscle, from which they were initially isolated and extensively studied (see [1] for a review). Recently, they have also been found in higher vertebrate's muscle [2,3], however at a somewhat lower concentration (approx. 0.05 mM).

Whereas considerable information is available on their primary and tertiary structure, on their physicochemical properties and on their evolution, nothing is known about their function. Their strong (K_d= approx. 10^{-7}M) and specific binding of two Ca^{2+} ions per mole [4,5] consequently has attracted much attention. It has been shown, however, that in spite of extensive sequence isologies with TN-C**, the myofibrillar Ca^{2+}-sensitizing factor [6], parvalbumins do not exhibit functional properties similar to those of the latter protein [7,8]. As many processes other than contraction, also in muscle cells, are actually controlled by the free Ca^{2+} ion concentration, it therefore appeared worthwhile to examine if parvalbumins could be detected in tissues other than muscle and thus a relation be established between the presence of these proteins and a cellular activity different from contraction.

Such an investigation, extending earlier work by Gosselin-Rey [9], was conducted here on the rabbit by means of a sensitive and quantitative immunological technique. It clearly shows that parvalbumins indeed are muscular proteins but that they are not present in all types of muscle, eliminating their direct participation in the contractile process itself. Complementary experiments, conducted mostly on frog muscle, furthermore indicate that parvalbumins are authentic sarcoplasmic proteins, and that their small size and lack of phosphate in all probability does not result from an artifact of their preparation.

Finally, total Ca^{2+} concentration and Ca^{2+}-binding activity were measured in the sarcoplasm. Whereas there is a good correlation between Ca^{2+} content and parvalbumin concentration in skeletal muscle from hake as well as from rabbit, a large excess of Ca^{2+} over parvalbumin (and even over soluble Ca^{2+}-binding proteins) is found in heart and in smooth muscle.

2. Materials and methods

2.1. *Materials*

Rabbits (*Lepus crawshayi*) and hakes (*Merluccius merluccius*) were obtained from the local market; frogs (*Rana esculenta*) were provided by the Laboratoire de Physiologie Animale, Faculté des Sciences, Montpellier. The different tissues were treated

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**Abbreviations: TN-C, troponin C; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetate; HAI, passive hemagglutination inhibition; Tris, Tris (hydroxymethyl) aminomethane; SR, sarcoplasmic reticulum; Palb, parvalbumin. Enzymes; Alkaline phosphatase, orthophosphoric monoester phosphohydrolase (EC 3.1.3.1)

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immediately after anesthesia and killing.

Rabbit and hake (pI = 4.36) parvalbumins, prepared as described previously [2,10], were supplied by Dr J.-F. Pechére. Antisera were obtained after repeated injection of protein emulsions in Freund's adjuvant to rabbits (anti-hake 4.36 parvalbumin) or to sheep (anti-rabbit parvalbumin).

Alkaline phosphatase from *E. coli* was kindly provided by Dr J. Attias.

2.2. *Methods*

Sarcoplasm was obtained by spinning muscle, segmented into pieces and overlayed with paraffin coloured by Sudan III, at 145 000 *g* during 1 hr.

The distribution of parvalbumin was studied in rabbit tissue extracts kept at 65°C during 2 min at pCa 4, and then centrifuged to remove the bulk of denatured protein. Under these conditions, the immunological reactivity of parvalbumin is unaltered, as shown by micro complement fixation (Baron, G. and Demaille, J., unpublished); its quantitative estimation in the supernatant was carried out in Microtiter plates by passive hemagglutination inhibition (HAI). Sheep red blood cells were coated with pure antigen using glutaraldehyde [11]. Buffer, antigen and antibody controls were run in parallel with antigen-coated cells, bovine serum albumin-coated cells and cells reacted with glutaraldehyde alone. This method allowed the determination of 0.25 picomol (3 ng) of parvalbumin in a final volume of 0.1 ml. Different dilutions were used in at least 6 different determinations, and the maximal error is given with the mean value.

Disc electrophoresis in 12% polyacrylamide gel at pH 8.9 was performed as previously described [10]. Protein was determined using the biuret [12] or the Lowry [13] method. Total Ca^{2+} was measured by atomic absorption spectrophotometry, using an apparatus Model 290 from Perkin-Elmer and 0.25% strontium diluent [14].

Assay for calcium-binding proteins was based on the competitive-binding Chelex 100 method of Briggs and Fleishman [15], as described by Oldham et al. [16]. Sarcoplasm was first dialyzed *vs* the assay buffer (60 mM KCl, 2 mM $MgCl_2$, 30 mM imidazole (HCl) pH 6.70) containing 2 mM EGTA, then *vs* the buffer without EGTA. Total Ca^{2+} concentration of 10–40 μM (at a total protein concentration of approx. 20 mg/ml) was lowered to 1–5 μM by serial 2-fold dilutions to 1/256 in the assay buffer. From percentage ^{45}Ca binding measurements made on dilutions in which less than 20% of the ^{45}Ca was bound [16], the figure corresponding to 0.1 ml undiluted sarcoplasm was calculated, and represents a rough relative estimate of the Ca^{2+}-binding proteins concentration.

3. Results and discussion

3.1. *Tissue distribution of parvalbumins*

The distribution of parvalbumins was studied in rabbit tissues using the sensitive and quantitative HAI technique, with the results shown in table 1. It was thus found that white and red skeletal muscles of rabbit contain similar amounts of parvalbumin (30–40 μM), whereas its amount in cardiac and smooth muscle is negligible, as had also been observed for the pike [9]. Parvalbumin concentration in diaphragm muscle is 10 times lower than in other skeletal muscles. Among the other organs, only brain does contain significant amounts of parvalbumin.

The figures in table 1 are obviously minimal, due on one hand to possible losses in the preparation of the heat stable proteins and, on the other hand, to the use of a specific antiserum vs the parvalbumin isolated from skeletal muscle (mostly white), which could not, perhaps, cross react with other hypothetical parvalbumins present in different tissues.

In spite of these limitations, the results show that, in all probability, parvalbumins are primarily muscular proteins, yet that they are not involved either in the contractile process itself (being absent from cardiac and smooth muscle), or in the steps of an ubiquitous major metabolic pathway such a glycogenolysis or lipolysis (as they are absent from liver). Predominance of aerobic or anaerobic metabolism is also irrelevant (as shown by skeletal muscle, heart and liver content). On the contrary a direct correlation exists between the presence of parvalbumins and a voluntary type of muscular activation, in contrast to spontaneous activity and hormonal regulation of contraction. The fact that, among all other tissues, brain is the only one to contain significant amounts of parvalbumin, is perhaps, not unrelated to this parallelism and suggests that parvalbumins might be connected in some way, as yet to

Table 1
Distribution of parvalbumin in rabbit tissue

Tissue	Parvalbumin content (μmol/kg fresh tissue)
Muscle	
White skeletal muscle	
– Psoas	32.5 ± 7.5.
– Longissimus dorsi	31.7 ± 7.5
Red skeletal muscle	
– Soleus	32.5 ± 7.5
– Cruris	40.0 ± 8.3
Diaphragm	3.1 ± 0.3
Cardiac muscle	< 0.01
Smooth muscle	
– Uterus	< 0.01
– Bladder	< 0.01
– Small intestine	< 0.01
Brain	0.15
Spleen	0.03
Kidney	0.02
Ovary	0.02
Mesenteric adipose tissue	< 0.01
Lung	< 0.01
Liver	< 0.01
Erythrocytes	< 0.01
Serum	< 0.01

Unless otherwise stated, all steps were carried out at + 4°C. Rabbit organs were removed immediately after death, washed and ground. 1.5 volumes of 0.1 mM $CaCl_2$, pH 7.0, were added, and the suspension was homogenized for 1 min at full speed in either a Waring Blendor or an Ultra-Turrax. The homogenate was centrifuged for 30 min at 14 000 *g*. The supernatant was brought to pH 7.0 with 0.1 N NaOH, heated to 65°C under vigorous stirring over a 2 min period, and immediately cooled to 4°C in an ice bath. The flocculent suspension was centrifuged for 30 min at 14 000 *g*, and the supernatant assayed for total protein and parvalbumin.

be defined, with the proper functioning of the nervous control of muscle contraction.

3.2. *Native state and localization of parvalbumins in muscle*

The yield in parvalbumins from fresh frog muscle was compared by disc electrophoresis for three different extraction procedures: direct collection of muscle juice, extraction with 1.5 vols (v/w) of the usual medium (0.3 M sucrose, 10 mM triethanolamine (HCl) pH 7.8) and extraction with 1.5 vols (v/w) of this buffer containing 10 mM sodium pyrophosphate to inhibit phosphatases [17]. The results depicted in fig.1a indicate that before, as well as after, extensive dialysis *vs* water, the two major parvalbumin bands (pI 4.50 and 4.88 [10]) were present in comparable amounts and with the same electrophoretic mobility in the three preparations. The minor acidic components [10] were more difficult to analyze on these gels. But such components could easily be examined by electrophoresis of the corresponding fraction from hake muscle [4]. Fig.1b shows that their electrophoretic mobility was not modified after overnight incubation at 38°C in presence of *E. coli* alkaline phosphatase (in 1 M Tris (HCl) pH 8.0, E/S = 1). These minor components therefore are not simply a phosphorylated form of the major parvalbumin which is known to be devoid of phosphate [4]; their amino acid composition, also, is different from that of the major component, and accounts for their lower isoelectric point .

These experiments clearly show that parvalbumins are present in muscle at least to a major extent, as genuine sarcoplasmic proteins of mol. wt 12 000, and furthermore that their obtention as low mol wt entities does not result from an artifact of their preparation, which involves a long and extensive dialysis of the crude extract during which proteolytic attack by cathepsins or dissociation from a multisubunit system could possibly occur. There is no evidence either, from these experiments, that the native form of parvalbumins could correspond to a phosphorylated protein, as has been suggested [18]. In fact, it is now likely (Demaille, J., unpublished) that the relation which has been suggested between parvalbumins and P-acceptor protein results from an artifactual situation.

3.3. *Calcium content of sarcoplasm*

Total Ca^{2+} and parvalbumin concentrations, and Ca^{2+}-binding activity at pCa 5–6, were measured in sarcoplasm from hake skeletal white muscle and rabbit skeletal, heart and smooth muscle, with the results shown in table 2.

In spite of a much higher parvalbumin content in hake muscle, the two high affinity Ca^{2+}-binding sites of parvalbumins can account for the whole (or almost) sarcoplasmic Ca^{2+} in rabbit as well as in hake skeletal muscle.

On the contrary, cardiac and smooth muscles,

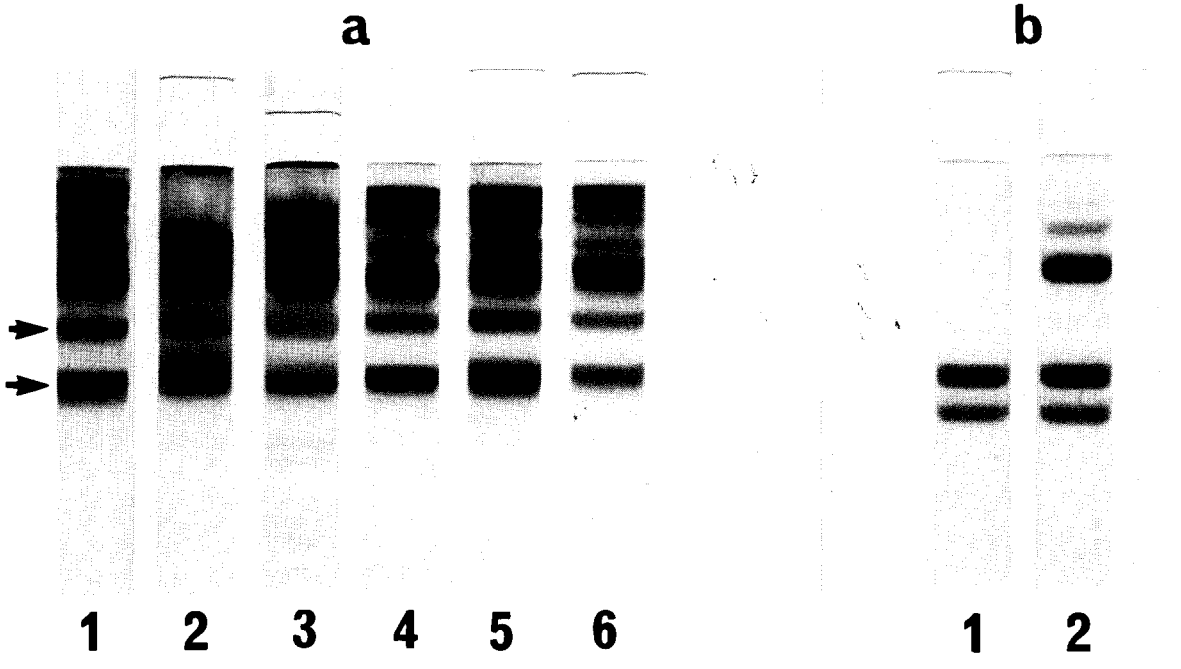

Fig.1. Native state of parvalbumin in muscle. (a): Disc electrophoreses of frog myogen. Samples 1 (550 μg protein), 2 (400 μg protein) and 3 (270 μg protein) were obtained respectively by ultracentrifugation and collection of muscle juice, by extraction with 0.3 M sucrose, 10 mM triethanolamine (HCl) pH 7.8, and by extraction with this buffer containing 10 mM sodium pyrophosphate. Samples 4 (240 μg), 5 (130 μg) and 6 (175 μg) correspond respectively to 1,2 and 3, after extensive dialysis vs water and elimination of the precipitated globulins. The bands corresponding to the two major parvalbumins [10] are indicated by an arrow. (b): Disc electrophoreses of hake minor parvalbumins (54 μg) before (sample 1) and after (sample 2) incubation with *E. coli* alkaline phosphatase, which forms the upper bands.

Table 2
Sarcoplasmic Ca^{2+} and Ca^{2+}-binding proteins

Muscle	Total protein (mg/ml)	Palb. (μM)	Palb. Ca^{2+}-binding capacity (μM)	Total Ca^{2+} (μM)	Ca^{2+}-binding ('%' ^{45}Ca bound/0.1 ml)
Hake white muscle	48	960 ± 110	1920 ± 220	1340	450
Rabbit – Psoas	75	57 ± 17	114 ± 34	90	26
– Soleus	73	82 ± 20	164 ± 40	180	31
– Diaphragm	38	7.5 ± 1	15 ± 2	240	23
– Heart	39	< 0.01	< 0.02	830	13
– Stomach	64	< 0.02	< 0.04	1640	13

Sarcoplasm, obtained by ultracentrifugation (see Methods) was diluted with 2 vol 0.25 M sucrose and spun again under the same conditions. The clear supernatant was assayed for Ca^{2+}, protein and parvalbumin. A single parvalbumin was assumed to be present in rabbit muscle. In hake muscle, minor palbs were 180 times less reactive than the major palb in the HAI test and did not interfere significantly. Hake total palb was obtained from the concentration of the 4.36 palb (680 ± 80 μM) multiplied by 1.41 to account for the minor components [10]. Parvalbumin Ca^{2+}-binding capacity was calculated on the basis of the high affinity sites only (2 Ca^{2+} atoms/mol palb). The whole stomach was used in this experiment (Mucosal components, especially mucins [20], may interfere with total Ca^{2+} and Ca^{2+}-binding).

which lack parvalbumins, contain millimolar Ca^{2+}, and there is no evidence of a soluble Ca^{2+}-binding protein in such a concentration that it could play a role similar to parvalbumins in skeletal muscle. These tissues (heart, smooth muscle and also fish red muscle [9]) exhibit common properties: low or negligible parvalbumin content, scarce SR and abundant mitochondria, which might substitute for parvalbumin by their energy-linked Ca^{2+} transport and storage [19]. Diaphragm muscle is intermediate between heart and fast skeletal muscle.

In conclusion, parvalbumins appear to be rather specifically present in fast skeletal muscles, which contain an extensive sarcotubular system, and rather few mitochondria, and are activated by a single nerve impulse in a Ca^{2+}-requiring process. They are able bind all (or almost) the sacroplasmic Ca^{2+} in these muscles.

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