Experientia 40 (1984), Birkhäuser Verlag, CH-4010 Basel/Switzerland

Parvalbumin, an intracellular calcium-binding protein; distribution, properties and possible roles in mammalian cells

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Key words. Parvalbumin; calcium-binding protein; muscle, mammalian.

Introduction

Calcium is involved in many biological functions and in most types of cell motility. Among the best-investigated calcium-dependent processes are excitation-contraction coupling in muscle, glycogen metabolism, membrane permeability, transmission of nerve impulses, release of neurotransmitters, cytoplasmic streaming, cell division, and secretory mechanisms.

Disturbance of calcium homeostasis can lead, for example, to defective bone mineralization (osteomalacia and rickets), renal failure, malignant hyperthermia, and parturient paresis in ruminants, and may have a pathophysiological role in the progression of muscular dystrophy. Cancer cells have been found to have abnormally large levels of calcium-binding proteins. It is thought that this may lead to a permanent activation of DNA synthesis without an extracellular calcium surge serving as a trigger. In this context it is of interest that a tumor-specific, Ca^{2+}-binding protein, oncomodulin, not present in normal fetal and adult tissues, has been discovered in rat hepatomas and other tumor cells[61].

The mechanism of action of calcium is different from that of other metal ions but similar to the regulatory action of cyclic nucleotides. They initiate multiple metabolic effects by binding to the regulatory subunit of cyclic AMP-dependent protein kinases which in turn modulate a number of additional enzyme activities. By analogy to the cyclic AMP system, it has been proposed that calcium represents another intracellular 'second messenger'[75]. Binding to various calcium binding proteins such as parvalbumin, calmodulin, S-100-proteins, vitamin D-dependent calcium-binding proteins and troponin-C (the calcium binding subunit of troponin), results in an amplification of the calcium signal and in analogy to the cyclic AMP signal, initiates a number of calcium-dependent mechanisms.

Parvalbumin, together with these proteins belongs to a homologous family of Ca^{2+}-binding receptors[54]. This is based on amino acid sequence data combined with a crystal structure analysis of carp parvalbumin (pI = 4.25).

Although these proteins are structurally related, owing to their common evolutionary origin[37], their cellular and intracellular distributions are quite different, indicating different physiological functions.

Calmodulin has been found to be involved in a variety of cellular activities[63] and troponin-C, the Ca^{2+}-binding subunit of troponin, is responsible for the calcium sensitivity of muscle contraction[26]. Vitamin D-dependent calcium-binding proteins are possibly required for calcium transport or to act as an intracellular calcium buffer system[80]. S-100 proteins[50] are a family of acidic proteins primarily found in glial cells of the brain but recently they have also been found in other tissues. Their physiological function is still unknown.

A class of calcium-binding proteins containing the modified amino acid, γ-carboxyglutamic acid[32] are involved in blood coagulation and bone formation. Their synthesis requires vitamin K, hence these proteins have been termed the vitamin K-dependent proteins.

Parvalbumins are small water-soluble proteins originally believed to be present only in skeletal muscles of lower vertebrates and which seemed to disappear in the course of evolution with the transition to terrestrial life. Previous reviews about parvalbumin have focussed mainly on the structural aspects of this protein in the muscles of lower vertebrates[23, 54, 82]. Although considerable information has been gathered in the past concerning the primary and secondary structures as well as calcium-binding properties of muscle parvalbumin from fish and amphibia, only limited evidence for its biological significance could be obtained.

This review will summarize the biochemical properties of parvalbumins from mammalian muscle and non-muscle cells, describe its cellular distribution and try to explore its physiological function. It will be shown that parvalbumin is always present in distinct populations of cells in some tissues where it is most probably involved in more specialized Ca^{2+}-dependent physiological processes. Quite in contrast, calmodulin is found in all eukaryotic cells, and has multiple regulatory functions in them.

Structural aspects

The existence of acidic proteins in extracts of frog muscle, made at low salt concentrations, the so-called low molecular weight 'albumins', was reported as early as 1934[24]. Later on, similar proteins were also found in muscles of several lower vertebrates[54]. Because of their small size and high water-solubility these proteins are now referred to as parvalbumins, although a functional relationship to serum albumin does not exist.

Parvalbumins from lower and higher vertebrates share common properties; they are all acidic, monomeric proteins with a molecular weight of approximately 12,000.

Isolation

Traditionally, parvalbumins from skeletal muscles of lower and higher vertebrates have been purified by ammonium sulfate fractionation, followed by gel filtration and anion exchange chromatography[33, 67]. Often heat treatment of the muscle extract was applied, which leaves most Ca^{2+}-binding proteins in solution, followed by the final purification of parvalbumin by 2 consecu-

tive anion exchange chromatography steps[10,43,78]. However, when the parvalbumin concentrations are considerably lower than those in the muscles of fish, amphibia or small mammals, as for example in human muscle[44] and in most non-muscle tissues[4], application of high-performance liquid chromatography on reverse phase supports is required for the isolation of parvalbumin[5,6].

Compared to the more conventional purification procedures the isolation by HPLC has the advantage of being much faster, and gives higher protein recoveries and homogeneity. This HPLC method has also been applied for the isolation of human parvalbumin when only limited amounts of starting material were available[7]. This method is suitable for the simultaneous isolation and quantification of parvalbumin and Ca^{2+}-binding proteins from various muscle and non-muscle tissues[6] and this is illustrated in figure 1. Nanomole quantities of parvalbumin can be detected and separated from calmodulin, troponin-C and S-100 proteins, which are often present in the same tissue extracts.

Amino acid compositions

Amino acid compositions of parvalbumins are generally characterized by a high number of glutamic acid, aspartic acid and phenylalanine, with little or no histidine, proline, cysteine, methionine, tyrosine or tryptophan. As a result of this unbalanced amino acid composition, parvalbumins show a very low absorbancy at 280 nm and a characteristic ultraviolet absorption spectrum owing to the high phenylalanine content.

Table 1 summarizes the amino acid compositions of the currently known muscle and non-muscle parvalbumins of higher vertebrates. Whereas the rabbit and rat muscle parvalbumins are rather similar (see also comparison of their sequences in table 2) except for the presence of one proline in the rabbit parvalbumin (none in rat), the chicken protein differs in its content of histidine, arginine, proline and tyrosine.

The rat brain parvalbumin is indistinguishable from its muscle counterpart[5] by its Mr (12,000), isoelectric point

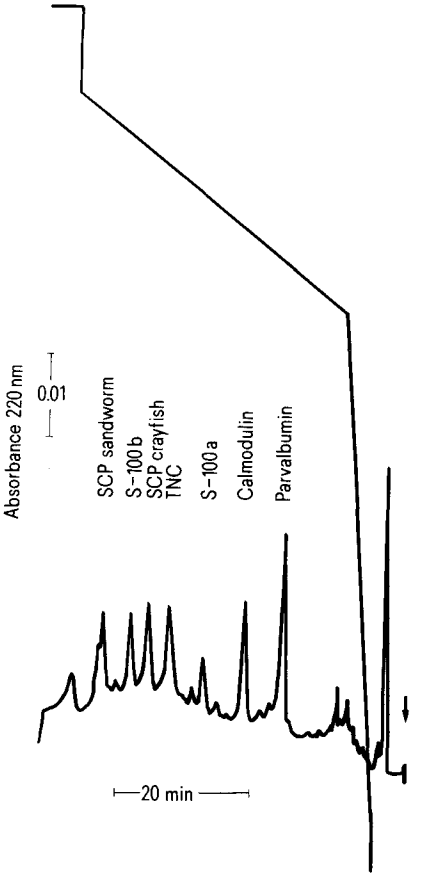

Figure 1. Separation by reverse-phase HPLC of parvalbumin from calmodulin, troponin-C, the sarcoplasmatic Ca^{2+}-binding protein from crayfish (SCP-CF) and the soluble Ca^{2+}-binding protein from sandworm (SCP-W) in an acid buffer system. For experimental details see ref.[6]. Amounts applied ranged from 5–25 µg of protein.
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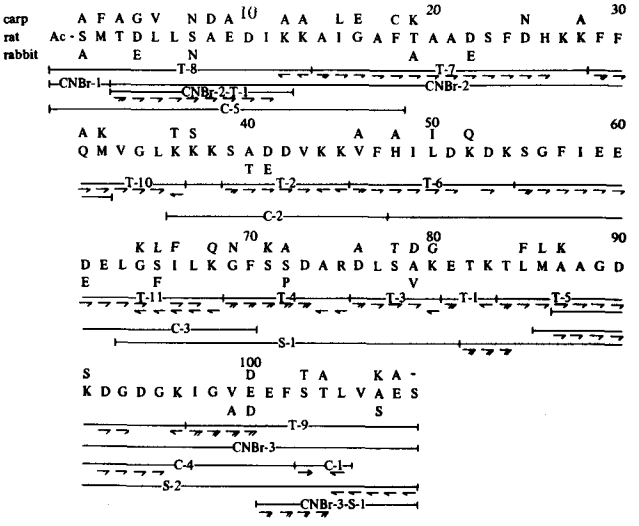
Table 1. Amino acid composition of parvalbumins

Amino acid	Rat brain	Rat muscle	Rabbit muscle	Chicken muscle	Mouse muscle
Lysine	15.5	16	16	13.2	16.0
Histidine	2.2	2	2	1.1	1.9
Arginine	1.0	1	1	2.2	1.0
Aspartic acid	14.6	14	12	11.8	13.8
Threonine	5.2	5	5	4.8	4.2
Serine	10.6	11	8	7.6	8.1
Glutamic acid	9.5	8	13	11.6	12.4
Proline	0	0	1	2.6	0.9
Glycine	9.4	9	9	9.9	9.2
Alanine	11.4	11	11	12.5	11.4
Valine	5.2	5	5	7.7	6.0
Methionine	2.4	3	3	2.8	2.1
Isoleucine	6.0	6	6	3.9	5.9
Leucine	9.6	9	9	7.8	9.3
Tyrosine	0	0	0	1.1	0
Phenylalanine	8.5	8	9	9.4	9.1
Tryptophan	0	0	0	0	0
Cysteine	0	0	0	0	0

Table 2. Amino acid sequence of rat muscle parvalbumin and comparison with rabbit and carp muscle (pI 4.25) parvalbumins. Amino acid residues (A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val. The residues which differ in rabbit and carp parvalbumin are indicated under the sequence of the rat protein. Reprinted by permission of the Eur. J. Biochem. *127* (1982) 381–389.



(pI 4.9), chromatographic behavior on the C-18 reverse phase column, Ca^{2+}-content (2 per molecule), and immunological properties. Brain and muscle parvalbumins also give tryptic peptide maps which are identical on analysis by HPLC and have the same amino acid composition (table 1). Mouse and human parvalbumins isolated by HPLC both show Mr and pI similar to rat muscle and brain parvalbumins, and identical hydrophobicities, demonstrated by the same retention times on a C-18 reverse phase column. However, their tryptic peptide maps are different from each other[7].

Muscles of lower vertebrates contain polymorphic forms of parvalbumin. For example in carp white muscle, 4 main components (II, III, IVa and IVb) have been found[38,67]. In contrast, in the muscles of mammals and man, only a single isotype of parvalbumin has been detected so far. However, 2-dimensional gel electrophoresis and HPLC of heat-extracts of a variety of muscle and non-muscle tissues of the rat has revealed the presence of several proteins with similar properties (Mr, pI and hydrophobicity) which are immunologically distinct but may still belong to the Ca^{2+}-binding protein family[4].

Calcium and magnesium binding

The Ca^{2+}- and Mg^{2+}-binding properties of parvalbumins from lower vertebrates have been extensively investigated[54,82]. Different association constants for the binding of Ca^{2+} and Mg^{2+} to parvalbumin have been reported. These differences were attributed to the ionic 'milieu' used, the diversity of methods applied and to the type of parvalbumin investigated. In general, parvalbumins have two high affinity sites for Ca^{2+}-ions ($K_{Diss.} = 0.1\text{–}4 \times 10^{-6}$ M) which also bind Mg^{2+} competetively. In vitro, under physiological conditions (1 mM Mg^{2+}; 80 mM K^+), and in vivo, in relaxed muscle ($Ca^{2+} \sim 10^{-8}$ M), parvalbumin binds 2 Mg^{2+} but no Ca^{2+}. Upon muscle contraction the intracellular Ca^{2+} level rises to 10^{-6}–10^{-5} M, resulting in a dissociation of the Mg^{2+} and subsequent binding of Ca^{2+}-ions. This indicates that parvalbumin is always present in a metal-bound form in vivo and that the exchange of Ca^{2+} with Mg^{2+} is of central importance for its physiological role in muscle and non-muscle tissues. Only a few metal analyses have been carried out on parvalbumins from higher vertebrates. The dissociation constants for Ca^{2+} have been determined for rabbit muscle parvalbumin [$K_{Diss.} \leq 10^{-6}$ M[10]] and chicken muscle parvalbumin [$K_{Diss.} \leq 10^{-6}$ M[43]] both in the absence of Mg^{2+}.

It has been reported that parvalbumin also binds ADP and ATP under physiological conditions and this may have also some functional importance[71].

Primary, secondary and tertiary structures

On the basis of the 3-dimensional structure of carp parvalbumin[64], it appears that the parvalbumins contain six α-helical regions, A to F, with the loops between the helices C and D and between E and F being the binding sites for the 2 Ca^{2+}-ions.

The amino acid sequences and the physicochemical properties indicate that parvalbumins from lower and higher vertebrates are isostructural with carp parvalbumin (pI 4.25). In addition, various spectroscopic and chemical studies show that the structure in solution is very similar to that in the crystal[55].

A comparison of the sequences of the rabbit[16,27] and rat muscle[8] parvalbumins, the only 2 sequenced parvalbumins from higher vertebrates, indicates 14 differences (table 2). The threonine at position 19, serine at 65 and 72 and alanine at 107 in the rat parvalbumin have not been encountered in previously sequenced parvalbumins from lower vertebrates. Two of the substitutions are limited to 'conserved' residues. This term defines positions where only different amino acids have been noted in the parvalbumins thus far sequenced. These include aspartic acid 61 and glutamic acid 100, each of which is either aspartic or glutamic acid in other parvalbumins. The remaining 12 substitutions are also on the surface of the protein and are considered to be at 'variable' time (asparagine in ray) that a hydrophilic residue has been noted in this position, other parvalbumins having either phenylalanine or leucine.

The secondary structures, predicted on the basis of the known amino acid sequences of rat[8] and rabbit[16,27] parvalbumin, are very similar. The most obvious difference is located at position 72–78, a sequence region starting with proline which is absent in the rat protein. This part of the protein lies between the Ca^{2+} and Mg^{2+}-binding CD and EF domains. More pronounced differences have been found in the predicted secondary structure of mammalian and carp (pI 4.25) parvalbumins, the most obvious being regions 64–72 and 80–85.

Position 64–72 belong to the D helix and region 80–85 is part of the E helix[56]. A helical structure is predicted on the basis of our analysis for amino acids 64–72 whereas for rabbit and rat parvalbumins rather extended structures are proposed. In contrast, helix E found in carp parvalbumin by X-ray diffraction studies is more strongly predicted for the mammalian proteins than for fish parvalbumin for which a mixed prediction pattern was obtained.

The rat muscle parvalbumin shows a higher degree of homology with rabbit muscle parvalbumin than with parvalbumins from lower vertebrates or other Ca^{2+}-binding proteins e.g. calmodulin, S-100 proteins, troponin-C or myosin light chains.

Functional roles of parvalbumin

Relaxation in skeletal muscle

Parvalbumins were originally isolated from frog and carp muscle, and were thought to be necessary 'as long as semipermeable membranes exist'[41]. Pechère and Focant[68] tested parvalbumin for a variety of possible enzymatic functions associated with glycolysis and found none. Based on the high affinity for Ca^{2+}, high tissue concentrations and structural homology with mammalian troponin-C, it was suggested that parvalbumin should be able to replace troponin in an in vitro ATPase assay system, but neither an influence on this enzymatic reaction nor an interaction of carp parvalbumin with troponin-I or tropomyosin could be demonstrated[48]. Parvalbumin (whether in the Ca^{2+}-bound or

Ca^{2+}-free state) did not affect the enzymatic activities of glycogen phosphorylase, phosphorylase kinase, phosphatase or protein kinase, and no phosphorylation of parvalbumin has been found under a variety of conditions[11]. Further suggestions for parvalbumin's physiological role, for example stimulation of the cyclic AMP phosphodiesterase[57,74] interaction with a membrane ATPase and parvalbumin being a buffer system for H^+ produced by ATP hydrolysis[69] have also been shown to be improbable.

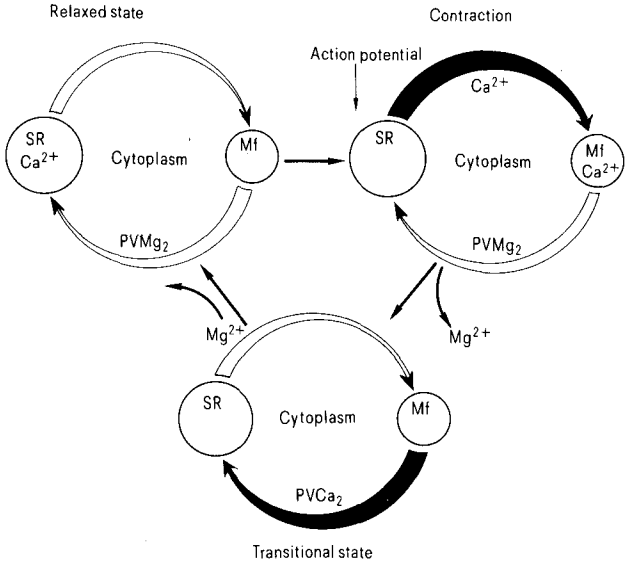
Comparison of the amino acid sequences of rabbit troponin-C and rabbit parvalbumin[27] also demonstrated that parvalbumin was not a proteolytic degradation product of the larger troponin-C molecule, a possibility arising from the degradation of dogfish troponin-C to a fragment of Mr 11,000 which cross-reacted with an antiserum to dogfish parvalbumin[45,62]. When it was found that parvalbumin had been retained throughout the entire range of vertebrate evolution, it was assumed that these proteins might play a general role in muscle contraction. Later on, however, a more specialized function in skeletal muscles was indicated by the observation that red muscles contain much lower concentrations of parvalbumin than white muscles and that there is non in most cardiac[1], chicken pectoralis[10,43], or smooth muscles. The more recent demonstration of parvalbumin not only in fish and amphibia, but also in mammalian muscles, raised the possibility that parvalbumin may act to regulate muscle contration and glycogen breakdown in a concerted manner. Instead, this role has now been attributed to calmodulin which is both a subunit of glycogen phosphorylase kinase[21] and seems, in complex with glycogen, glycogen phosphorylase and glycogen debranching enzyme to be (at least in part) directly associated with the myofibrillar structure[46].

Briggs[14] identified parvalbumin as the soluble relaxing factor of muscle. Subsequently it was shown that fragmented sarcoplasmic reticulum could deplete parvalbumin of Ca^{2+} and parvalbumin could take up Ca^{2+} from the myofibrils (troponin C)[10,34]. This is illustrated in a scheme modified from Pechère et al.[70]. Although the affinity of parvalbumin for Ca^{2+} is higher than that of troponin-C, the rate of Ca^{2+}-binding to troponin-C exceeds that of parvalbumin. Hence upon release from the sarcoplasmic reticulum, Ca^{2+} will first interact with troponin-C and subsequently activate the myosin ATPase. In a further step, Ca^{2+} is removed by parvalbumin and finally taken up again by the sarcoplasmic reticulum leading to the relaxation of the muscle fiber[29,35,40,70]. The computer study of Gillis et al.[35] indicated that at the time relaxation is complete, a large fraction of the Ca^{2+} is still bound to parvalbumin and will return into the sarcoplasmic reticulum during the post-contractile (recovery) period. This is in agreement with the autoradiographic studies of Winegard[81], who demonstrated that calcium is present in the intermyofibrillar space a few seconds after a tetanus.

This model for the role of parvalbumin in relaxation has recently been disputed. It has been concluded, also on the basis of kinetic measurement, that parvalbumin cannot be directly involved in the rapid decay of tension after single muscle twitches. This is essentially because of the slow Ca^{2+} on-rate (in relaxed muscle fibers Mg^{2+} is bound to parvalbumin and must be removed first) and also because of the slow Ca^{2+} off-rate (resulting from the high affinity of these sites for Ca^{2+})[52].

Thus, despite a similar experimental approach there was disagreement about the in vivo Ca^{2+}-binding properties and functional role of parvalbumin in skeletal muscle. Therefore a different approach to this problem was undertaken combining immuno-histochemical, physiological and biochemical methods. Using monospecific antisera against homogeneous rat muscle parvalbumin[5,17], parvalbumin immunoreactivity in the extensor digitorum longus muscle of newborn rats was found to appear on postnatal days 3 to 4[18], coincident with changes in contraction properties, in particular the decrease in the relaxation time after a twitch or a tetanus[20,25].

In adult rats, anti-parvalbumin serum stains extensor digitorum longus extrafusal muscle fibers with at least 5 different degrees of intensity[18] (fig. 2a). In the soleus



Scheme of calcium-movement in fast skeletal muscle fibers during the contraction and relaxation cycle. In the *relaxed state*, Ca^{2+} is in the sarcoplasmic reticulum (SR) and parvalbumin (PV) saturated with Mg^{2+}. Upon arrival of the action potential Ca^{2+} is released from the sarcoplasmic reticulum store and first taken up by the ca^{2+}-specific sites of the troponin-C (the Ca^{2+}-binding subunit of troponin) leading to *contraction*. Following dissociation of Mg^{2+}, PV removes Ca^{2+} from the myofibrils (Mf) (troponin-C) leading to a *transitional state* where Ca^{2+} (bound to parvalbumin) is localized in the sarcoplasm of the muscle cell. In a final step returning to the *relaxed state*, sarcoplasmic reticulum removes the Ca^{2+} from the parvalbumin and Mg^{2+} is rebound to parvalbumin.

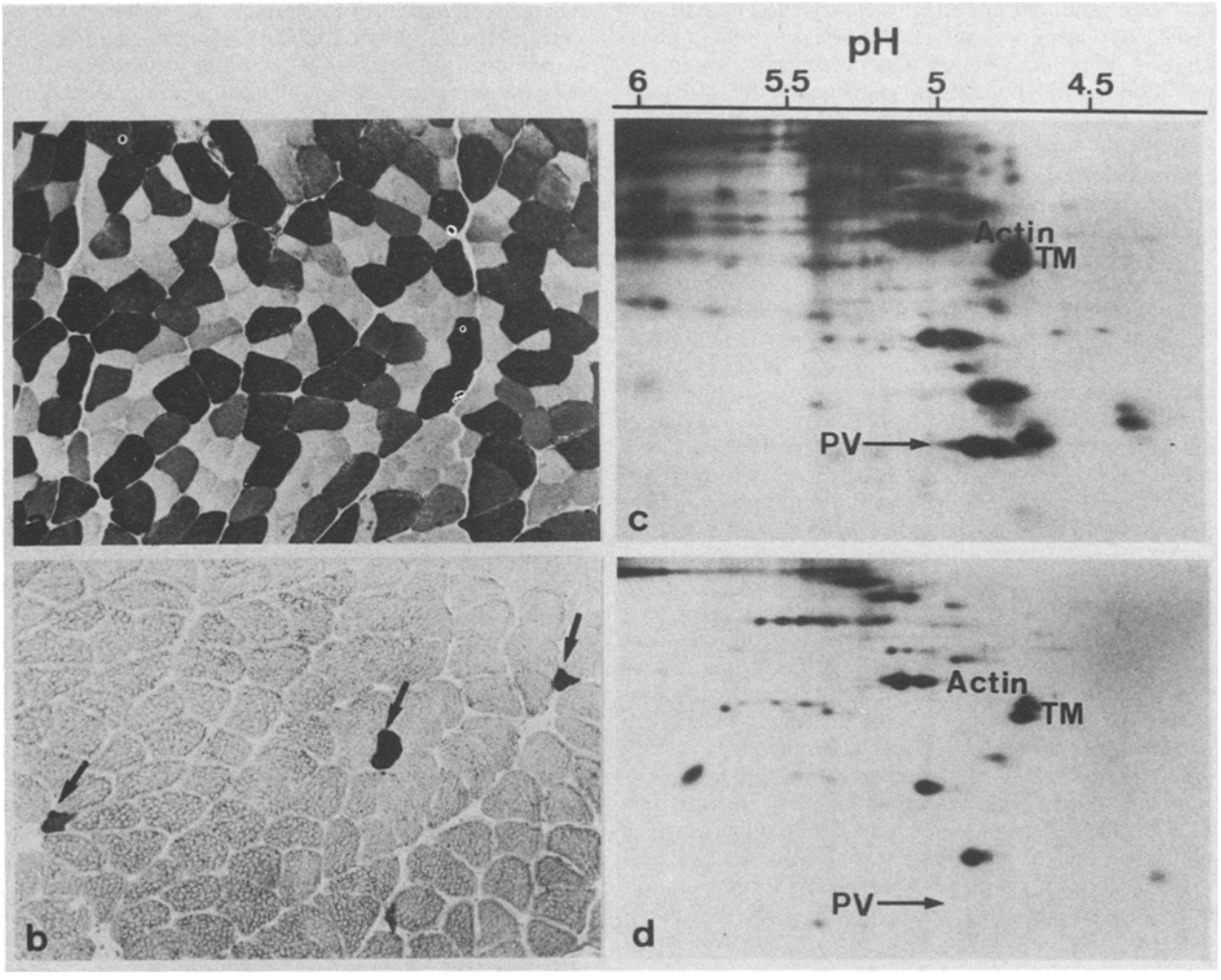


Figure 2. Immunohistochemical localization of parvalbumin. (a) Transverse-section through the belly of the extensor digitorum longus muscle of an adult rat (fast contracting/fast relaxing). Checkerboard appearance of the immunostaining with muscle fibers stained with at least 5 degrees of intensity. IIB fibers stained strongly: IIA moderately and type I remained unstained. (b) Transverse-section through the belly of the ipsilateral soleus (slow contracting/slow relaxing) of the same animal incubated with anti-parvalbumin at the same time. Only a few fibers are stained (arrows). Reprinted by permission from J. biol. Chem. *259* (1984) 5189–5196.
Electrophoretic analysis of typed single muscle fibers. Two-dimensional polyacrylamide (15%) gels of a single type I fiber and type IIB fiber from rat muscle. (c) Type IIB fiber shows a high concentration of parvalbumin (PV), whereas (d) in the type I fiber parvalbumin could not be detected. Proteins were [^{14}C] labelled and visualized by fluorography. Parvalbumin was identified by its pI of approximately 5 and Mr of 12,000; TM, tropomyosin; PV, parvalbumin. Reprinted from Proc. natl Acad. Sci. USA *79* (1982) 7243–7247.

muscle the parvalbumin immuostaining pattern is much less conspicuous (fig. 2b). The parvalbumin immunostaining pattern correlated with the histochemical fiber typing by staining serial section for myofibrillar ATPase. The muscle fibers can thus be identified as type I (slow-twitch), and type IIA and type IIB, which are subgroups of the type II (fast-twitch) fibers. The type IIB fibers show the strongest immunoreactivity towards the parvalbumin antiserum and can be further differentiated into 2 subgroups showing slightly different levels of intensity. The type IIA muscle fibers are moderately stained with 3 distinct degrees of intensity. Type I muscle fibers which have a relatively poorly-developed sarcoplasmic reticulum and a slower relaxation time, lack parvalbumin immunoreactivity.

The different staining intensities presumably correspond to differences in the concentration of parvalbumin within these muscle fibers and may be associated with the continuum of relaxation properties. The existence of a sub-population of type II fibers, of a dynamic continuum of muscle fiber types, and a wide spectrum of contraction and relaxation times is in agreement with these immunohistochemical results.

The extra-ocular muscles and the intrinsic laryngeal muscles, both displaying the highest intrinsic speed of shortening and the shortest twitch half-relaxation times, showed strongest parvalbumin staining only in the fast-contracting fibers belonging to the global portion of the lateral rectus muscle of the eye muscle and in the thyreoarytenoideus lateralis musle of the larynx. The slow tonic fibers in the orbital layer of the extra-ocular muscles and the slow-twitch fibers of the thyreoarytenoideus medialis muscle are unreactive[18].

Cardiac muscle fibers and smooth muscle cells are not

labelled by parvalbumin antiserum. In muscle spindles only one of the 2 nuclear chain fibers is parvalbumin reactive whereas the 2 bag fibers are not. It is probable that the parvalbuminpositive intrafusal nuclear chain fibers relax faster than to nuclear bag fibers.

These immunohistochemical results are supported by an electrophoretic analysis of typed single fibers of rat muscle[44]. Highest concentrations of parvalbumin are found in the type IIB (fast-twitch, glycolytic) muscle fibers and the protein is undetectable in type I (slow-twitch, oxidative) fibers (fig. 2c, d).

If parvalbumin is involved in muscle relaxation, then it can also be expected to be homogeneously distributed throughout the sarcoplasm. The homogeneous distribution of parvalbumin in this immunohistochemical study of rat skeletal muscles is in agreement with earlier observations indicating that parvalbumins are sarcoplasmic proteins. No interaction of parvalbumin with other soluble or structural muscle proteins has so far been demonstrated in the muscles of chicken[43], carp[2], perch[3] and frog[36].

If parvalbumin is involved in the relaxation process of muscles, its distribution and concentration must fulfil a number of criteria. First, within any single species of animal more parvalbumin should be present in muscles known to have fast relaxation times than in muscles which relax slowly. Similarly, the parvalbumin content of individual muscles within each species should be related to their fiber type composition, with more parvalbumin present in muscles with a high proportion of fast-twitch (type II) fibers since these also relax faster than slow-twitch fibers.

Second, muscles of the same fiber type composition but from animals of different size, should contain more parvalbumin in the smaller animal. This derives from an argument presented by Hill[47] that animals of similar design, but different size, have very similar top running or swimming speeds. It therefore follows[49] that for the same running speed, small animals must move their legs to and fro much faster, and have muscles with much higher shortening velocities. This faster contraction time is, for obvious reasons, accompanied by shorter relaxation times. Third, in any one species, muscles which have similar fiber type compositions but different relaxation times might be expected to have correspondingly different parvalbumin contents.

In table 3 parvalbumin concentrations were directly compared with the contraction and relaxation properties of a variety of muscles of several mammalian species including man.

The quantitative analysis performed by HPLC on reverse phase supports showed the highest parvalbumin concentration, 4.9 g/kg of wet weight, in the superficial portion of the gastrocnemius muscle of the mouse. This was the smallest animal investigated, and has the highest speeds of muscle contraction and half relaxation

Table 3. Correlation of parvalbumin content of various muscles with physiological data

Muscle	Fiber type composition (%)	Contraction time (msec) Motor units	Whole muscle	Half-relaxation time (msec) Motor units	Whole muscle	Parvalbumin (g/kg) wet wt
Mouse (0.02–0.035 kg b.wt)						
Gast. (sup.)	IIB, 100					4.9
Soleus	I, ≈ 50; IIA, ≈ 50	I and IIA, 37–7	I and IIA, 17.7; I, IIA, 20.9	I and IIB, 31–10	I and IIA, 22.5	0.01
EDL	I, ⩽ 1; IIA and IIB, ⩾ 95	IIA and IIB, 8.7	IIA and IIB, 6.9	IIA and IIB, 8.6	IIA and IIB, 7.1	4.4
TA	I, ⩽ 1; IIA and IIB, ⩾ 95					4.8
Rat (0.25–0.55 kg b.wt)						
Gast. (sup.)	IIB, 100					3.3
Soleus	I, 85; IIA, 15	I, 37; I, 38; I, 26–44, IIA, 15–20	I and IIA, 34	I, 49; I, 51; I, 45; IIA, 11	I and IIA, 49	⩽ 0.004
EDL	I, ⩽ 5; IIA and IIB, ⩾ 95	IIA and IIB, 11; IIA, 9.9; IIB, 8.9	I, ≈ 30; IIA and IIB, 13; IIA and IIB, 10	IIA and IIB, 10.5; IIA, 10.2; IIB, 8.4	IIA and IIB, 8; IIA and IIB, 9	2.4
TA	I, ⩽ 5; IIA and IIB, ⩾ 90					2.7
EOM	I, 20–30; IIA and IIB, 70–80		IIB, 5		IIA and IIB, 6.3	1.0
RB	IIB, 100					1.8
Guinea pig (0.7–1.0 kg b.wt)						
Masseter	IIA, 100					0.08
Soleus	I, 100		I, 82		I, 113.8	⩽ 0.007
Sartorius	IIB, 100					0.25
Fast muscles	I, 4–18; IIA and IIB, 96–82		IIA and IIB, 19–22		IIA and IIB, 19–21	
Horse (500–700 kg b.wt)						
Deep gluteal	I, 22; IIA, 43; IIB, 35	Unknown		Unknown		⩽ 0.001
Man (60 kg b.wt)						
Vastus	I, ≈ 50; IIA and IIB, ≈ 50					⩽ 0.001
Triceps	I, ≈ 50; IIA and IIB, ≈ 50					⩽ 0.001
EHB		I, 79, IIA and IIB, 43	I and IIA, 57	I, 85.7; IIA and IIB, 50	I, IIA, and IIB, 42	
1st DI		I, IIA, and IIB, range 100–30; IIA and IIB, ≈ 40		I, IIA, and IIB, 45; range 60–20		

Gast. (sup.), gastrocnemius (superficial portion); EDL, extensor digitorum longus; TA, tibialis anterior; RB, retractor bulbi; EHB, extensor halucis brevis; 1st DI, first dorsal interosseus (hand). Reprinted from Proc. Natl. Acad. Sci. USA 79 (1982) 7243–7247. For details see this reference.

times (see table 3). The same muscles in the rat contain less parvalbumin (3.4 g/kg) and sartorius of the guinea pig only 0.25 g/kg. Since these 3 muscles contain only IIB fibers, it is clear that IIB muscle fibers of the guinea pig contain 20 times less parvalbumin than those of the mouse. Similarly, comparing extensor digitorum longus muscle of the mouse and the rat, both muscles consisting of approx. $\leqq 5\%$ type I and $\geqq 95\%$ type II fibers, the parvalbumin concentration in the mouse muscle is roughly twice as much as in the rat.

Horse and human muscles have very low concentrations of parvalbumin (≤ 1 mg/kg wet weight). The direct comparison of 3 different muscles of the rat (table 3), gastrocnemius, extensor digitorum longus and soleus, demonstrates a wide range of parvalbumin concentrations within a single species depending on the fiber type composition of the individual muscle. Masseter, soleus and sartorius muscles of the guinea pig have the useful characteristic of being homogeneous type IIA, I and IIB muscles, respectively. The quantitative HPLC analysis (table 3) revealed that the soleus muscle (pure type I) contains only ≤ 0.007 g parvalbumin/kg, masseter (pure type IIA) 10 times as much (0.08) and the sartorius (pure type IIB) the highest parvalbumin concentration (0.25), consistent with the known differences in the relaxation properties.

Denervation is known to affect the contractile properties of fast-twitch muscles such as the extensor digitorum longus muscle of the rat[58]. Major changes occurred over a time period from 2–6 days after denervation, affecting mostly the twitch time-to-peak and half-relaxation, both of which are prolonged[28].

The parvalbumin concentration in the denervated extensor digitorum longus muscle is 20% lower than in the control muscle[44].

In another investigation, the central stump of the nerve to the soleus (slow-twitch) muscle of the rat was connected to the distal portion of the nerve to the fast-twitch extensor digitorum longus muscle. This procedure can be expected to cause the cross-reinnervated fast muscle to acquire the mechanical, biochemical and immunological properties of a slow twitch muscle[15]. These experiments[65] revealed a decrease in the parvalbumin concentration of the rat fast-twitch extensor digitorum longus muscle by a factor of 2–3 after cross-innervation with the soleus nerve.

Together, these results demonstrate a close correlation between parvalbumin content and relaxation speed and are in full agreement with the hypothesis that parvalbumin is a relaxing factor in fast-twitch skeletal muscle fibers.

Parvalbumin in non-muscle tissues

Parvalbumins are not restricted to contractile cells but are also found in carp brain[38], various non-muscle tissues of the pike[39], chicken brain[43] and brain, spleen, kidney and ovary of the rabbit[1]. The analysis of a variety of heat treated non-muscle extracts of the rat by 2-dimensional gel electrophoresis[4] and by HPLC reveals the presence of several proteins, similar in their Mr, pI and hydrophobicity to muscle parvalbumin, but distinct from calmodulin, S-100 proteins, vitamin D-dependent Ca^{2+}-binding proteins and troponin-C. Therefore several independent criteria have to be applied to identify genuine parvalbumins with more certainty. Extracts from bone, teeth, skin, testis, prostate, seminal vesicles, ovary, cerebrum and cerebellum contain a protein identical to the muscle parvalbumin in its Mr of 12,000, its pI of approximately 5 and its hydrophobicity. Several proteins occurring close to muscle parvalbumin on 2-dimensional gels, or which have a similar hydrophobicity may either represent isoparvalbumins (as found in lower vertebrates) or more likely may belong to the assorted group of parvalbumin-like proteins. Rat kidney, however, contains a prominent protein with an identical pI but lower Mr compared to parvalbumin, which crossreacts with the anti-parvalbumin serum. This protein appears to be localized in part of the distal tubule and the proximal collecting duct. Thus immunologically similar but biochemically distinct proteins may exist, and their structural and functional relationship to parvalbumins remains to be established.

There are a few other tissues, e.g. spleen, thyroid gland, mammary gland, lachrymal gland, tendon and adipose tissue where faint parvalbumin-immunoreactivity can be visualized by the dot-immuno-binding assay. This suggests that these non-muscle tissues may also contain trace amounts of parvalbumin or parvalbumin-like proteins.

(a) *Central and peripheral nervous system.* Parvalbumin from rat brain is the only non-muscle parvalbumin which has been isolated and characterized[5]. Its biochemical and immunological properties are found to be indistinguishable from the muscle counterpart.

Antiserum against rat muscle parvalbumin selectively recognizes a subpopulation of neurons in the brain of the rat, and astro- and oligodendroglia remain unstained[17]. In all parvalbumin-positive neurons the soma, dendrites and axons are homogeneously labelled whereas nuclei are mostly unreactive. Parvalbumin-immunoreactive neurons are present in all areas of the neocortex (fig. 3) and in all layers except layer I. In the cerebellum, all Purkinje cells show strong parvalbumin immunoreactivity. In the molecular layer, some basket and stellate neurons are labelled whereas the granular cells display no immunostaining. By contrast, calmodulin occurs in both Purkinje and granular cells of the cerebellum[59]. In the hypothalamus, immunoreactive neurons are widely scattered except for some clustering in the lateral preoptic area and in the premamillary nuclei. In the olfactory bulb periglomerular cells in the glomerular layer are parvalbumin-positive and in the hippocampus, single immunoreactive neurons, probably basket cells, are observed within the pyramidal layer. In the peripheral nervous system (fig. 4a, b) parvalbumin-immunoreaction is found in large cells of the spinal ganglia and their peripheral processes. Only a certain percentage of the peripheral nerve fibers are parvalbumin-positive. Very small immunoreactive neurons and positive punctate structures can be seen in lamina IIB (Rexed) of the dorsal horn of the spinal cord. Labelled terminals surrounded motor-neurons in the ventral horn, and heavily immunoreactive myelinated axons coursed through the dorsal funiculi. In spinal ganglia

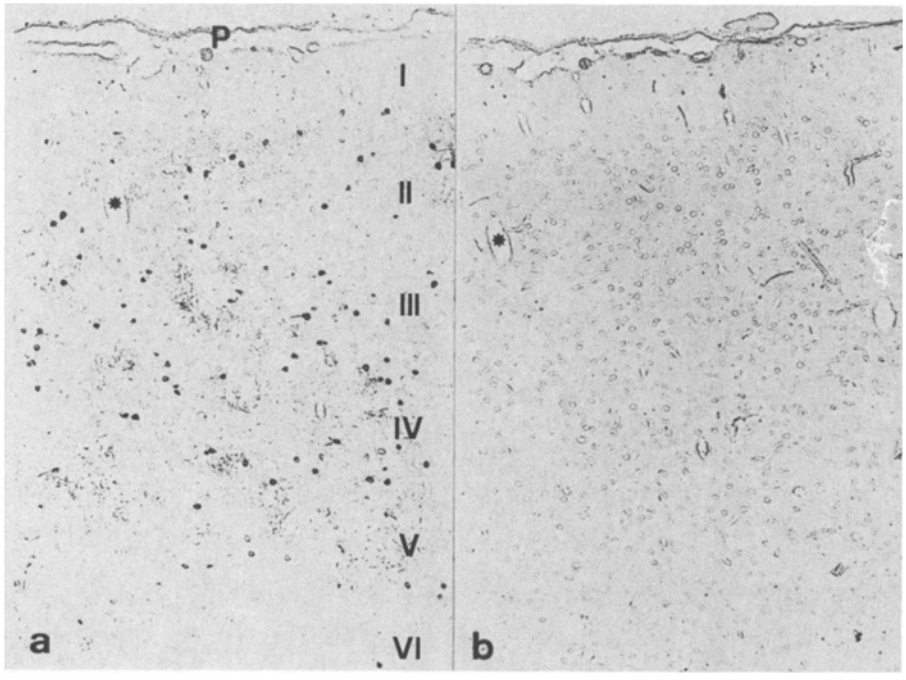


Figure 3. Localization of parvalbumin in rat brain. (a) Sagittal section of an adult rat brain stained with parvalbumin antiserum. Myriads of small interneurons with processes branching in close proximity to the cell body are visualized. These neurons are randomly scattered in laminae II-VI with a preferential localization in laminae II and IV; P, Pia mater; Asterisk points to a blood vessel which is a useful landmark for comparison with (b). (b) Adjacent serial section to (a) incubated with the preimmune-serum of the same animal. No structures are immunolabelled. Reprinted by permission from Nature *293* (1981) 300–302, Macmillan Journals Limited.

immunolabelling is restricted to large cells and in the peripheral nerves to large diameter axons.

Parvalbumin has also been shown to be present in brain cell cultures derived from 14-day-old embryonic mice[72]. Cells showing typical neuronal morphology were intensely stained by anti-parvalbumin serum and the same cells were also labelled by the antiserum directed against the neuronal $\gamma\gamma$-enolase but not by an anti S-100 serum, a marker for glial cells. In all developmental stages investigated (0–28 days in culture) approximately 90% of these neurons are labelled by the anti-parvalbumin serum. In rat brain, only a subpopulation of neurons (approximately 20%) are parvalbumin-immunoreactive. This discrepancy may arise from incomplete terminal differentiation of these neurons in vitro or by the possibility that only the parvalbumin-containing neurons survive under in vitro conditions.

The localization of parvalbumin in a distinct sub-population of neurons raises the question of its functional role in the central nervous system. It has been noticed that similarities exist between the distribution of parvalbumin-positive neurons and those containing the inhibitory neurotransmitter γ-aminobutyric acid (GABA). The parvalbumin-positive Purkinje, basket and stellate cells in the cerebellum, the basket cells in the hippocampus and periglomerular cells in the olfactory bulb all use GABA as their neurotransmitter[31]. However, the confirmation of a preferential association of parvalbumin with GABA-containing neurons awaits further studies. Parvalbumin is one of a few neuron-specific antigens; others are the neurotransmitter synthesizing enzymes, olfactory marker protein, $\gamma\gamma$-enolase and some neuron-specific surface antigens[12, 13, 76]. Some of these antigens occur only in local subgroups of neurons segregated in certain brain areas; the function of most of them is unknown. Although it is not possible to classify parvalbumin-positive neurons as members of a homogeneous group of neurons, parvalbumin may be a marker for neurons displaying Ca^{2+} action potentials, as in the case of Purkinje cells. Ca^{2+} in micromolar quantities plays a critical role in the release of neurotransmitter substances such as nor-adrenaline, dopamine and acetylcholine at chemical synapses[22].

The arrival of the action potential at the pre-synaptic terminal causes a transient depolarisation and an associated increase in Ca^{2+}-permeability. The resulting influx of Ca^{2+}-ions initates neurotransmitter release[22]. Following repolarization, the rate of transmitter release falls off with a time constant of about 1 msec, as Ca^{2+} returns towards its resting levels[53]. However, it may take about 10 msec to extrude the Ca^{2+} that enters during depolarization[9]. Thus it was suggested that intracellular proteins with a high affinity for Ca^{2+} must be responsible for the rapid fall in Ca^{2+} after repolarization.

In analogy to the involvement of parvalbumin in the fast relaxation process in muscle which occurs on roughly the same time scale, parvalbumin in the brain seems to be a good candidate for being a factor controlling free Ca^{2+} in the neuroplasm in the distinct subpopulation of neurons where strong immuno-reactivity has been demonstrated. This would imply that parvalbumin is a soluble factor in these neurons as it has been reported to be in muscle, but its precise intracellular distribution is at present under investigation. Calmodulin has also been implicated in mediating Ca^{2+}-dependent synaptic functions and neuronal excitability[9, 22, 53].

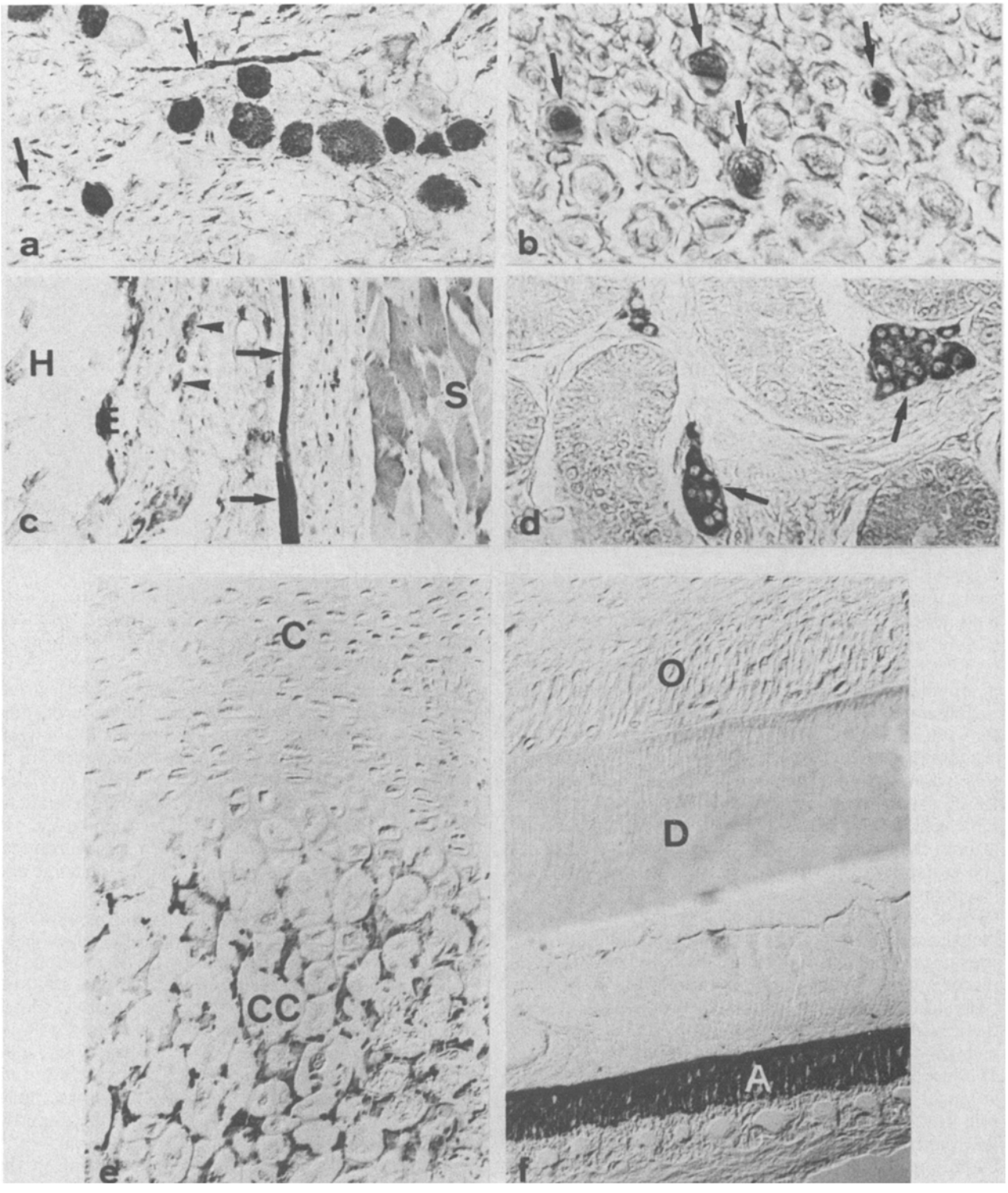


Figure 4. Immunohistochemical localization of parvalbumin in non-muscle cells. (a) Longitudinal section of a spinal ganglion at L_1. The largest cells (50 µg as determined with the aid on an ocular micrometer) are parvalbumin positive. The same is true for the axons belonging to the proximal and distal processes arising from these bipolar ganglion cells (arrows). (b) Cross section of the ischiatic nerve. The parvalbumin positive axons (arrows) are followable in the peripheral nerve and can be shown to arise from intrafusal muscle fibers. They are the largest diameter axons in the peripheral nervous system. (c) Section through the skin of the rat body. Epidermis (E), hairs (H) and composite glands (arrow heads) are parvalbumin negative. A thin 2-fiber thick layer of muscle tissue in the reticular layer of the cutis stains exceptionally strongly with parvalbumin-antisera (arrows). The subcutis (S) display lightly staining muscle fiber bundles. The nuclear staining is probably an artefact. (d) Testis of a 7-day-old rat immunostained with parvalbumin antisera. The reaction sites occur only in the Leydig cells (arrow) lying in the interstitium. (e) Longitudinal section of the vertebral column of a rat fetus on day 15 of gestation. The calcification center (CC) of the vertebral body stains with parvalbumin-antisera whereas the cartilage (C) does not. (f) Longitudinal section of the root of an interior incisor tooth of a 22-day-old female rat. The ameloblasts (A) display parvalbumin immunoreactivity in their cytoplasm. The odontoblasts (O) and dentin (D) are unstained. Reprinted by permission of J. biol. Chem. *259* (1984) 5189–5196 .

An involvment of parvalbumin in the Ca^{2+}-dependent axoplasmic transport in the nerve[66] or other Ca^{2+}-dependent processes have also to be considered.

(b) *Endocrine glands.* The function of the testis as an endocrine gland resides mainly in the Leydig cells, which produce androgenic steroid hormones, principally testosterone. These cells stain strongly with anti-parvalbumin serum, and parvalbumin has also been demonstrated to be there by biochemical means[4]. The secretory activity of the Leydig cells is under the control of gonadotropic hormones of the hypophysis, such as the luteinizing hormone. Maximum luteinizing hormone stimulation of testosterone production in the Leydig cells of rat testis can only be obtained in the presence of Ca^{2+}[51]. It has also been established that the presence of Ca^{2+} is a prerequisite for full stimulation of steroid synthesis by adrenocorticotropin in the adrenal gland.

The 2 endocrine glands, the ovary and testis, contain a genuine parvalbumin, but its cellular localization has only been investigated in the testis. There, the Leydig cells are strongly immunostained with the parvalbumin antiserum (fig. 4d) and thus some connection with hormone production or secretion seems possible.

(c) *Mineralizing tissues.* A protein similar to parvalbumin in its biochemical and immunological properties occurs in mineralizing tissues[4]; however, at present it has not yet been isolated in a pure form. In addition, mineralizing tissues also contain osteocalcin[42, 73] and osteonectin[79], both proteins with similar hydrophobicities[7]. The physiological role of osteocalcins is not understood; there are indications that osteocalcin synthesis may be regulated by Vitamin D[60]. Osteonectin, a bone specific protein linking mineral to collagen, is thought to initiate active mineralization in normal skeletal tissue[79].

Using anti-parvalbumin serum for the immunohistochemical study, parvalbumin-immunoreactivity has been demonstrated in the calcification center of bone[19, 80] (fig. 4e) and in the ameloblasts in the teeth (fig. 4f), the cells which elaborate the enamel matrix. The odontoblasts, the dentin-forming cells which secrete a predentin matrix consisting of type I collagen, acidic glycoproteins and Ca^{2+}-binding phosphoproteins, did not stain with anti-parvalbumin serum. From these results it may be inferred that parvalbumin (possibly in concert with other Ca^{2+}-binding proteins) plays a crucial role in the initiation of calcification, the mechanisms of which have recently been reviewed[30, 77].

Concluding remarks

The range of functions of the calcium-binding receptor protein parvalbumin has been seriously underestimated in the past. Originally thought to be of major importance only in the muscles of fish and amphibia, parvalbumin is likely to be involved also in the relaxation of fast-twitch muscle fibers in mammals. A rewarding approach will be to investigate the precise physiological function of parvalbumin in various non-muscle cells where, in contrast to calmodulin, it has always been detected in only a few distinct cell types. Although at the moment most of the results concerning the structure and distribution of parvalbumin in non-muscle cells must be considered to be mainly phenomenological, the presence of parvalbumin in a restricted population of neurons in the central nervous system, its presence in specific cells in endocrine glands (for example the Leydig cells of the testis) and its location in some cell types only in mineralizing tissues strongly suggest that parvalbumin may also be involved in the regulation of the activity of some types of neurons, in hormone production and in the initiation of calcification.

Acknowledgments. I am indepted to Drs A. Rowlerson, E. Jenny and C. C. Kuenzle for valuable discussion, my wife for typing this manuscript and the Swiss National Science Foundation (Grant 3.185–0.82), the Sandoz-, Fritz Hoffmann-La Roche- and Wilhelm Sander-Stiftungen for financial support.

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0014-4754/84/090910-12$1.50 + 0.20/0

Full Papers

Mapping of restriction enzyme cuts by a new two-dimensional procedure*

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Summary. A new procedure has been worked out to establish restriction maps. The method is fast, does not in general require labeled DNA and has been applied to map the linear palindromic rDNA of *Physarum* with the restriction enzyme *Bst*EII.
Key words. Physarum; ribosomal DNA; restriction; method, two-dimensional; gel electrophoresis.

The establishment of a restriction map for a given piece of DNA is a rather tedious procedure. With unlabelled material, recourse is often made to several digests, whereby elution of fragments from gels is followed by a subsequent cleavage. If DNA pieces are radioactively labelled at both ends, cut once and the 2 fragments separated, restriction maps can be constructed from partial digests[13]. Here we describe a new 2-dimensional procedure. The method entails separating partial digests on a gel, transferring them to a solid support, eluting and digesting them to completion with the same enzyme and separating the fragments in a second gel. The transfer of DNA fragments from one gel to another is simple and quick, and has recently been described in detail in a very different context[7]. This novel procedure has been successfully applied to establish a *Bst*EII restriction map of *Physarum* ribosomal DNA. This rDNA is a linear palindrome of 60 kb size, not integrated in large chromosomes[11, 15].

Material and methods. rDNA was purified from *Physarum polycephalum* plasmodia (strain M_3 CVIII) as previously described[4]. Restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII were obtained from Bethesda Research Labs; *Bst*EII was obtained from Boehringer Mannheim. Digestion conditions were those used in the procedures described by the manufacturers. Electrophoresis of DNA restriction fragments was performed in agarose gels in the tris-acetate buffer of Hayward and Smith[10]. Borate buffer is not suitable[7]. The gels were stained with ethidium bromide (0.5 µg/ml) for 30 min, and photographed under UV illumination using a Kodak No. 23A Wratten filter.

Two-dimensional electrophoresis and recovery of DNA fragments were performed according to Chen and