Parvalbumin in rat kidney

Purification and localization

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The Ca^{2+}-binding parvalbumin has been purified for the first time from rat kidney. Its biochemical and immunological properties were indistinguishable from the muscle counterpart. By immunohistochemical methods parvalbumin was localized in part of the distal tubule and proximal collecting duct, similar to the vitamin D-dependent Ca^{2+}-binding protein, calbindin-28_K. Parvalbumin was found to be independent of the vitamin D status of the animal since its concentration remained unchanged in kidney extracts of normal, rachitic and vitamin D-replete rats. Both proteins may be involved in the regulation of intracellular Ca^{2+} in kidney.

Ca^{2+} binding protein *Parvalbumin* *Vitamin D* *Calbindin-28_K* *(Kidney)*

1. INTRODUCTION

The Ca^{2+}-binding parvalbumin (PV) has been found in muscle and non-muscle tissues of the rat [1,2], including some major target organs for the hormonally active vitamin D metabolite, 1α,25-dihydroxycholecalciferol (1α,25-$(OH)_2D_3$). Vitamin D is known to enhance the Ca^{2+} transport across these tissues and concomitantly induces the formation of the vitamin D-dependent calcium-binding proteins, calbindin-10_K and -28_K [3–5] which are possibly involved in Ca^{2+}-transport/buffering functions also proposed for PV. It has been noticed that PV and calbindin-28_K coexist in the ameloblast of teeth, calcified cartilage of bone and in some neurons (e.g. Purkinje cells) of the cerebellum [1,2,6].

Here, we isolated for the first time PV from rat kidney, determined its biochemical properties, compared them with the muscle counterpart and determined its concentration and localization in kidneys of normal and rachitic animals to test if also the biosynthesis of PV (similar to those of calbindins) may depend on the vitamin D status of the animal.

2. MATERIALS AND METHODS

2.1. *Proteins*

PV from rat skeletal muscle [7], calmodulin from calf brain [8] and calbindin-$10/28_K$ [9] were isolated as described.

2.2. *Antibodies*

Antisera against rat muscle PV were raised in rabbits [1,2]. The antibody was purified from whole serum by ammonium sulfate fractionation and chromatography on a PV affinity column.

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Abbreviations: RIA, radioimmunoassay; calbindin-10_K/-28_K, vitamin D-dependent Ca^{2+}-binding proteins; HPLC, high-performance liquid chromatography; 1D/2D-PAGE, one-dimensional/two-dimensional polyacrylamide gel electrophoresis; 1α,25$(OH)_2D_3$, 1α,25-dihydroxycholecalciferol; PAP, peroxidase anti-peroxidase

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2.3. *Animals*

Vitamin D-deficient, vitamin D-supplemented and control animals were obtained from Hoffmann-La Roche [10,11]. The vitamin D deficiency was examined by: (i) X-ray analysis of the epiphyseal growth plates of the hindlimbs; (ii) determination of the plasma concentration of $1\alpha,25\text{-}(OH)_2D_3$; (iii) and disappearance of calbindin-10_K from rat intestine.

2.4. *Purification of kidney PV*

Kidneys from 3–6-month-old rats (SIV-50) were pooled and frozen immediately after being killed. Extraction was carried out at 4°C in 2 vols of 4 mM EDTA, pH 7.5, containing various protease inhibitors [8]. After homogenization (Virtis blender 4 × 15 s, full speed), and centrifugation (100000 × *g*) the supernatant was directly applied to an immune-affinity column (10 × 0.6 cm) prepared by coupling of anti-PV IgG to CNBr-activated Sepharose 4B (Pharmacia). After washing with phosphate-buffered saline (PBS, containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.3), followed by PBS containing in addition 1 mM EDTA and 1 M NaCl, PV was finally eluted with 3 M KI, 0.1 M Tris-HCl, pH 8. The protein was immediately dialyzed against H_2O (48 h) and lyophilized.

2.5. *HPLC*

Proteins were separated [1,7] on an Aquapore RP-300 column (4.6 × 30 mm, RP-8, 10 μm particle size, 300 Å pore size support) at a flow rate of 2 ml/min. Buffer systems: IA, 50 mM Tris-HCl, pH 7.5, 0.1 mM $CaCl_2$ (or 0.1 mM EGTA); IB; same as in A except 60% (v/v) acetonitrile.

2.6. *2D PAGE*

^{14}C-labeled proteins were analyzed as described [7,8].

2.7. *Immunoblotting*

Proteins were first separated on 1D-PAGE (15%) and then transferred onto nitrocellulose (Millipore) using an LKB apparatus with a constant voltage of 15 V for 16 h [12]. Antiserum against rat muscle PV (raised in rabbits) and peroxidase-coupled goat anti-rabbit IgG (Miles) were diluted 1:1000 and 1:1500, respectively. The affinity-purified antibodies were used in a concentration of 1 μg/ml. Final staining was carried out with chloronaphthol as a substrate.

2.8. *Immunohistochemistry*

Fü-Albino rats were anesthetized with sodium pentobarbital, perfused through the aorta with 500 ml of Bouin fluid, the tissue dissected out, dehydrated in graded ethanol and finally embedded in paraffin. The immunostaining was performed using the PAP method [1,2]. Antiserum against rat PV was diluted 1:1000 to 1:20000 in PBS and the affinity-purified antibodies in a dilution of 0.2 to 1 μg/ml, goat anti-rabbit IgG 1:5 (Nordic) and the PAP complex 1:200 (Sternberger-Meyer). Control incubations used preimmune sera or preabsorbed antisera.

2.9. *RIA*

PV was iodinated [13]. In short, 100 μl of the ^{125}I-labeled PV (3000 cpm) were mixed with 100 μl kidney extract (before and after heat treatment) and varying amounts of unlabeled antigen. After 3 h incubation at 37°C, polyethyleneglycol and γ-globulin were added and the solution centrifuged. Pellets were counted in an LKB γ-counter.

2.10. *Double-antibody solid-phase enzyme immunoassay*

Briefly, anti-PV IgG was coated onto the surface of microtiter wells (1 μg/well, micro ELISA plate, Dynatech) and further processed [14].

3. RESULTS

3.1. *Isolation of PV from kidney*

Conventional as well as HPLC techniques applied for the isolation of PV from muscle and brain [1,7] were unsuccessful. First, because of the high proteolytic activity in kidney extracts and second because even after 'final' purification, the protein fractions were still contaminated with 3–5 low-molecular-mass proteins (mainly cytochromes), some of them eluting with identical hydrophobicities to PV. Therefore kidney PV was purified on an immune-affinity column. Heat-treated extracts (30 min, 85°C) were directly applied onto an anti-PV IgG-Sepharose column. After several washing steps, homogeneous PV was eluted with 3 M KI, 0.1 M Tris-HCl buffer, pH 8.

Approx. 1 mg of protein was obtained from 1 kg tissue.

3.2. *Characterization of kidney PV*

3.2.1. 2D-PAGE

The EDTA extracts of rat kidney (after heat treatment) still contained approx. 30 distinct proteins with kidney PV clearly recognizable by its M_r of 12000 and pI of 4.9 (fig.1a). After purification on the immune-affinity column, PV was obtained in a homogeneous form (fig.1b) co-migrating with muscle parvalbumin (M_r 12000/pI 4.9, fig.1c).

3.2.2. HPLC analysis

Purified kidney PV when chromatographed in the presence of 1 mM $CaCl_2$ was eluted in a single peak at 44% acetonitrile (fig.2a, left) at the same position as muscle PV (fig.2b, left) but distinct from calmodulin (fig.2c, left). Kidney and muscle PVs were considerably shifted in their hydrophobicity when chromatographed in the presence

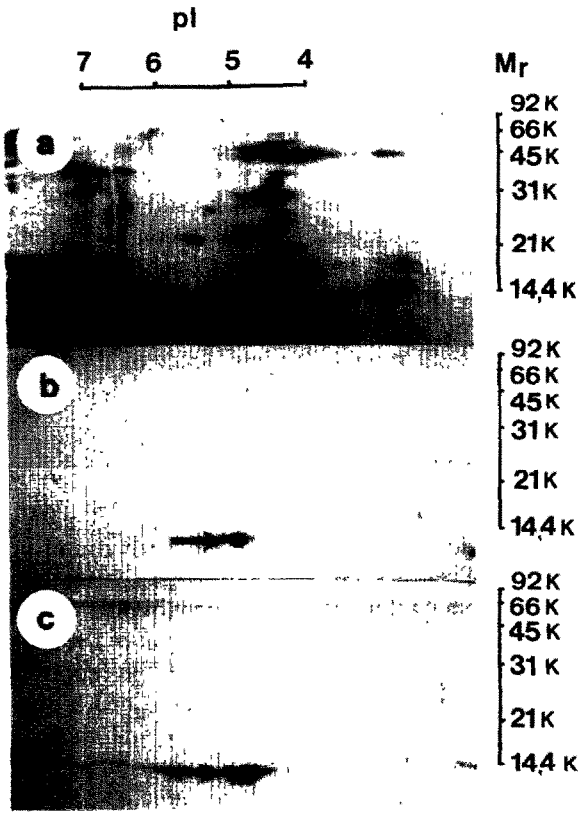

Fig.1. 2D-PAGE (15%) of (a) heat-treated extract from rat kidney (150 μg protein), (b) purified kidney PV (5 μg) and (c) comigration of kidney and muscle PVs (5 μg each). Proteins were ^{14}C-labeled by reductive methylation and visualized by fluorography. PV was identified by its M_r = 12000, pI = 4.9. Arrows show position of PV; arrowheads position of cytochromes *c*.

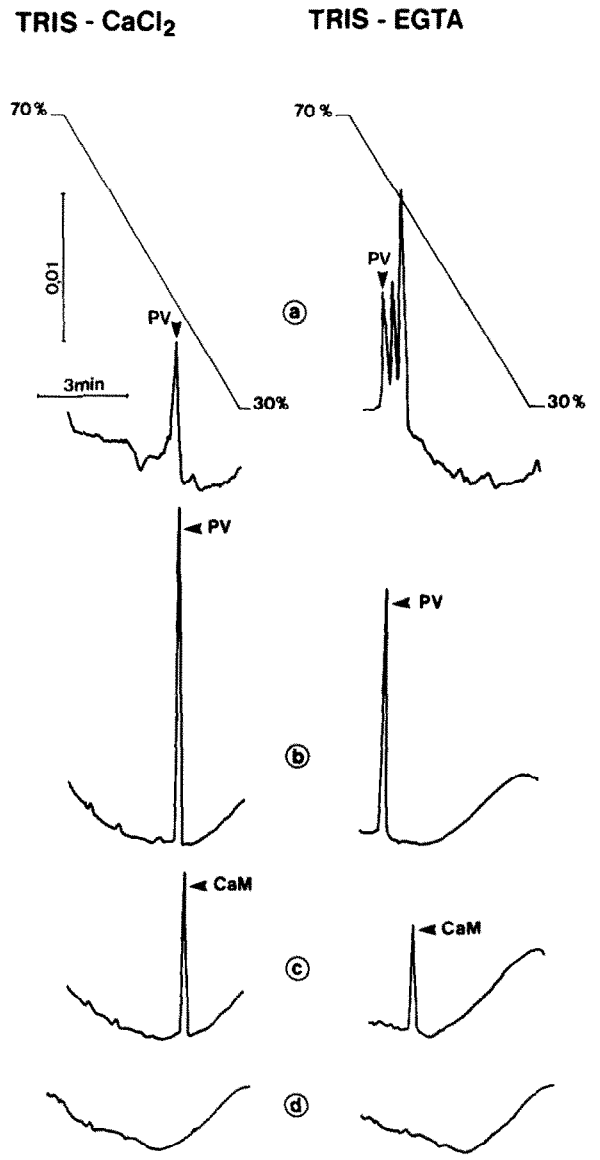

Fig.2. HPLC of (a) purified kidney PV (5 μg protein), (b) muscle PV (10 μg), (c) CaM (5 μg). Left, in the presence of 1 mM $CaCl_2$; right, with 1 mM EGTA. (d) Blanks. The gradient was 0–100% buffer B in 15 min. Only part of the chromatogram ranging from 30–70% buffer B is shown.

of EGTA and eluted at 64% acetonitrile. However, in the Ca^{2+}-free buffer system the kidney PV sometimes showed three closely spaced peaks (fig.2a, right). When analyzed on 2D-PAGE, only the peak eluting at the same position as muscle PV showed a protein spot (M_r 12000/pI 4.9). The other two peaks did not show any protein bands and most probably represent degradation products of kidney PV.

3.2.3. Peptide maps

Purified kidney PV was digested with TPCK-treated trypsin and peptides analyzed by HPLC. The peptide pattern of the kidney and muscle proteins were indistinguishable but different to those of calmodulin (not shown).

3.2.4. Immunoblotting

Kidney and muscle PVs (fig.3) were first separated on 1D-PAGE and then transferred onto a nitrocellulose sheet. The immunoreaction of anti-muscle PV serum with kidney and muscle PV demonstrated immunological identity (fig.3c,d). A parallel gel (fig.3a,b) was run with identical samples after ^{14}C labeling of the proteins by reductive methylation, and visualized by fluorography.

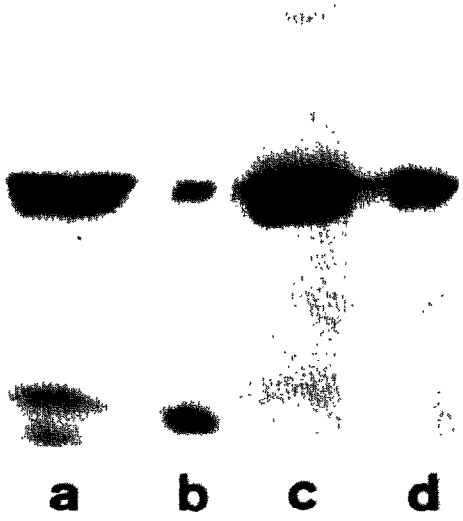

Fig.3. Fluorograms of 1D-PAGE (15%) of (a) kidney PV (1 μg) and (b) muscle PV (1 μg) and corresponding immunoblots (c,d).

3.3. *PV in normal and rachitic rats*

Immunoreactivity in rat kidney (fig.4a) was found in a portion of the tubular system, identifiable as part of the distal tubule and proximal collecting duct. The immunoreactivity was homogeneously distributed in the cytoplasm of these cells and also seemed to occur in the nuclei. No staining has been found in the kidney in controls, incubated with pre-immune- or pre-adsorbed antisera (fig.4b). Kidney extracts (after heat treatment) of normal, rachitic and vitamin D-supplemented (not shown) animals were analyzed for PV on 2D-PAGE. The intensity of the PV spot, recognized by its M_r of 12000 and pI of 4.9, remained unchanged in all extracts indicating an independence of PV from 1α,25-$(OH)_2D_3$ (not shown). Quantitative analysis of PV in these extracts was performed by RIA and a double-antibody immunoassay. PV concentrations (1 mg/kg tissue wet wt) remained unchanged when extracts (before and after heat treatment) of normal, rachitic and vitamin D-replete animals were analyzed confirming an independence of PV from 1α,25-$(OH)_2D_3$ as found above.

4. DISCUSSION

Besides muscle, trace amounts of PV-immunoreactive material have also been found in several non-muscle tissues of various species but isolation, characterization and anatomical localization have so far only been carried out in the central nervous system [1,2]. Previously we reported [15] that also rat kidney extracts contained a protein with an isoelectric point (pI 4.9) identical to PV but with a slightly lower molecular mass. This protein cross-reacted with serum directed against rat muscle PV. It was suggested [15] that this protein may either represent another as yet unknown kidney protein or may be a proteolytic fragment of PV, since kidney extracts are known to contain high proteolytic activities. By application of immune-affinity chromatography, a genuine PV could be isolated in small quantities of fresh and

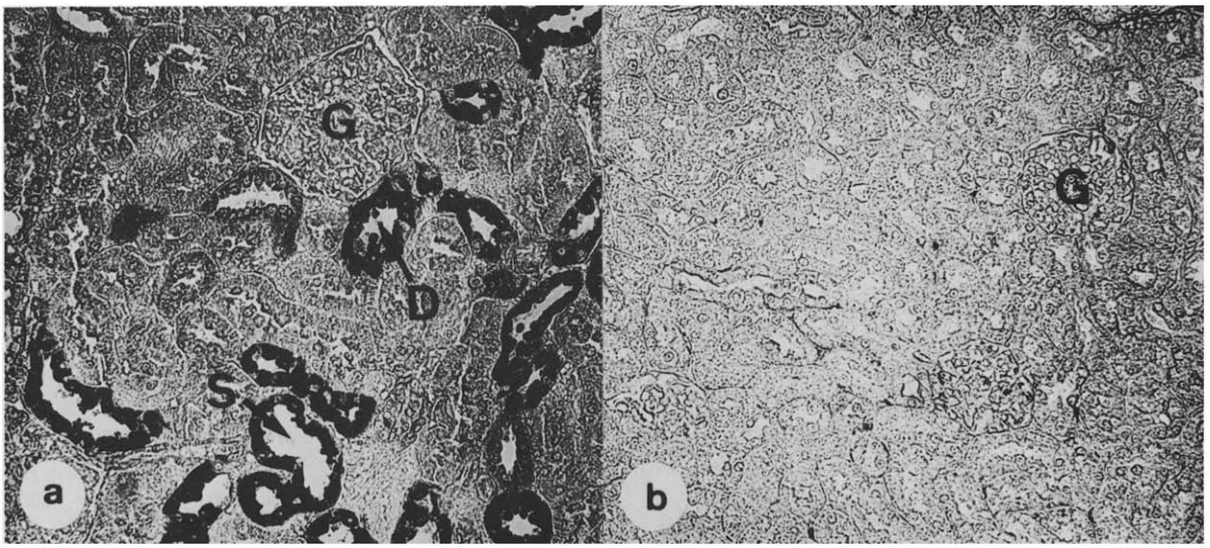

Fig.4. Immunohistochemical localization of PV in rat kidney. (a) Section through the kidney cortex of a normal adult rat incubated with PV antiserum. A portion of the tubular system, namely part of the distal tubule and proximal collecting duct was stained. (b) Control incubated with pre-immune serum. D, distal tubule; S, proximal collecting duct; G, glomerulus.

quickly frozen material. The lower molecular mass observed earlier probably resulted from limited proteolysis. This view is supported by the fact that PV in kidney extracts was degraded upon storage (even in the presence of various protease inhibitors). A direct comparison of the kidney PV with its muscle (or brain) counterpart revealed identities in molecular masses, isoelectric points, retention times on reverse-phase supports (in the presence and absence of Ca^{2+}) as well as in their immunological properties. The following reasons prompted us also to investigate a possible dependency of the biosynthesis of PV from vitamin D: (i) structurally homologous vitamin D-dependent, calbindin-10_K and -28_K, have been found in kidneys of chicken, several mammalian species including man [3–5,16–20]. These previous studies, however, produced some conflicting results on their vitamin D-dependence and the regulatory effect of their cellular expression; (ii) PV and calbindin-28_K co-exist in some neurons and ameloblasts of teeth [1,2]; (iii) PV and calbindin-28_K are thought to regulate intracellular translocation of Ca^{2+} or represent intracellular Ca^{2+} pools. Although PV is found in some vitamin D target tissues and is co-localized with calbindin-28_K, its synthesis in kidney as found in the cerebellum [11] seems to be independent from the vitamin D status of the animal.

PV immunoreactivity was found in a portion of the tubular system of the kidney cortex of a normal adult rat, identifiable as a part of the distal tubule and proximal collecting duct. A similar distribution has been described for calbindin-28_K. In the kidney of chicken, calbindin-28_K immunoreactivity was found in the distal convoluted tubule, the initial collecting tubule and early parts of the collecting tubule and similar localization have been found in other species [3–5,16–20]. Calbindin-10_K has also been found in mouse kidney [16,17] and immunoreactivity has been observed, e.g. in the rat nephron, with strongest reaction in cells of the distal convoluted tubules but also in the macula densa and collecting duct. Therefore kidney contains either calbindin-28_K as reported in rat [18] or even two calbindin-28_K and -10_K as found in mouse [16,17,19] whose synthesis seems to be dependent on $1\alpha,25\text{-}(OH)_2D_3$, but in addition, rat kidney also contains the Ca^{2+}-binding PV whose concentration seems unaffected by the vitamin D status.

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