Parvalbumin: calcium and magnesium buffering in the distal nephron

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Abstract
Parvalbumin (PV) is a classical member of the EF-hand protein superfamily that has been described as a Ca\(^{2+}\) buffer and Ca\(^{2+}\) transporter/shuttle protein and may also play an additional role in Mg\(^{2+}\) handling. PV is exclusively expressed in the early part of the distal convoluted tubule in the human and mouse kidneys. Recent studies in Pvalb knockout mice revealed a role of PV in the distal handling of electrolytes: the lack of PV was associated with a mild salt-losing phenotype with secondary aldosteronism, salt craving and stronger bones compared with a mild salt-losing phenotype with secondary aldosterone deficiency. A link between the Ca\(^{2+}\)-buffering capacity of PV and the expression of the thiazide-sensitive Na\(^{+}\)–Cl\(^{-}\) cotransporter was established, which could be relevant to the regulation of sodium transport in the distal nephron.

Properties and distribution of parvalbumin
Parvalbumin (PV) is a small protein (109 amino acids in most species; molecular mass ∼12 kDa) first isolated from carp muscle in 1973 [1] and belonging to the subfamily of cytosolic Ca\(^{2+}\) buffers in the superfamily of EF-hand proteins [2]. Proteins of the EF-hand family are characterized by a conserved, helix–loop–helix structural unit, which consists of two α-helices bridged by a Ca\(^{2+}\)-chelation loop. These proteins are involved in the regulation of many critical cellular processes including gene transcription, protein phosphorylation, nucleotide metabolism and ion transport [3–5]. Over 200 members of the Ca\(^{2+}\)-binding EF-hand superfamily have been identified so far in the human genome [2].

PV is characterized by a high affinity for Ca\(^{2+}\) (dissociation constant \(K_{D, Ca} \sim 5–10\) nM) and an intermediate affinity for Mg\(^{2+}\) (\(K_{D, Mg} \sim 30\) μM). Thus, the two functional metal-binding sites are so-called mixed (Ca\(^{2+}\)/Mg\(^{2+}\))-binding sites. These properties and the fact that the intracellular concentration of Mg\(^{2+}\) \(([Mg^{2+}]_{cyt}, 0.5–1\) mM) exceeds largely that of Ca\(^{2+}\) \(([Ca^{2+}]_{cyt}, 5–100\) nM) in basal conditions explain why PV-binding sites are (>80%) occupied mainly by Mg\(^{2+}\) in a resting cell. The remaining sites are either Ca\(^{2+}\)-bound or metal-free. When a stimulus induces a rise in [Ca\(^{2+}\)]\(_{cyt}\) levels, Mg\(^{2+}\) slowly dissociates from the binding sites and is replaced by Ca\(^{2+}\). Because of the slow Ca\(^{2+}\)-binding kinetics under physiological conditions, which mainly results from this prior Mg\(^{2+}\) dissociation, PV is referred to as a slow-onset Ca\(^{2+}\) buffer [2]. Binding of two Ca\(^{2+}\) ions to the Ca\(^{2+}\)-binding sites induces a rather insignificant conformational change [4]. Hence, PV is mostly considered a pure Ca\(^{2+}\) buffer, with little or no Ca\(^{2+}\) sensing and direct regulatory properties [6].

The expression of PV is remarkably restricted to a few cell types in the brain, skeletal and heart muscles, parathyroid glands and kidney [7–9]. In the central nervous system, PV is highly expressed in inhibitory GABAergic interneurons, e.g. in the cortex in chandelier (axo-axonic) and basket cells. These cortical neurons play an important role in controlling pyramidal cell excitability. The absence of PV in these cells is linked to increased drug-induced seizure susceptibility [10]. PV is also expressed in similar types of interneurons in the hippocampus (axo-axonic and basket cells) and cerebellum (stellate and basket cells) [7]. In the cerebellum, PV is additionally expressed in Purkinje cells.

In the kidney, PV is expressed in the epithelial cells lining a subset of tubules in the distal nephron (Figure 1). In the mouse and human kidneys, PV appears to be exclusively expressed in the early part of the distal convoluted tubule (early DCT, or DCT1), where it colocalizes with the thiazide-sensitive apical Na\(^{+}\)–Cl\(^{-}\) cotransporter (NCC).
The progression between the early and late part of the DCT is characterized by an abrupt transition from PV (early DCT) to calbindin-D28k (late DCT, or DCT2). Cells expressing calbindin-D28k also express NCC and epithelial Na+ channel (ENaC) [11, 12]. In the rat kidney, PV has been located in the thick ascending limb (TAL) of the loop of Henle, late DCT, connecting tubule (CNT) and intercalated cells of the collecting duct [13]. Immuno-cytochemical analyses revealed that in most tubular cells, PV shows a diffuse cytosolic pattern, with a signal sometimes enhanced along the basolateral membrane [12, 13].

PV is present in fast-contracting and fast-relaxing skeletal muscle fibres (e.g. extensor digitorum longus or tibialis anterior) [14]. The protein has also been detected in normal and in hyperplastic and adenomatous human parathyroid glands, with the strongest expression in chief cells and water clear cells. Of note, PV colocalizes with the parathyroid hormone (PTH) in the same cell types [8].

Role of PV in the distal nephron

The distal nephron plays a major role in the reabsorption of NaCl and the regulation of the final excretion of Ca2+ and Mg2+, under the influence of several hormones, including aldosterone, PTH and 1,25(OH)2-vitamin D3 [15]. Approximately 5% of the filtered load of NaCl is reabsorbed in the DCT, involving the thiazide-sensitive NCC on the apical side and the Na+-K+-ATPase and the ClC-Kb chloride channel on the basolateral side of the cells. The reabsorption of Ca2+ and Mg2+ in that segment is mediated by a complex interaction of different proteins, involving two specific members of the transient receptor potential (TRP) channel superfamily that have a distinct spatial distribution along the DCT (Figure 2). Approximately 5–10% of filtered Mg2+ is passively reabsorbed through the apical TRPM6 channel and basolateral active transport systems in the DCT1. Specific intracellular
Mg\textsuperscript{2+} carriers and basolateral extrusion proteins have not yet been identified. Apical TRPV5 channels (previously named epithelial calcium channel 1, ECaC1) mediate the passive entry of Ca\textsuperscript{2+} in the DCT2, before active, basolateral transport via the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger 1 (NCX1) and plasma membrane Ca\textsuperscript{2+}-ATPase 1b (PMCA 1b). In the DCT, TRPV5 colocalizes with the intracellular Ca\textsuperscript{2+} buffer/sensor calbindin-D\textsubscript{28K} \cite{16, 17}. Studies in rabbit CNTs have shown that calbindin-D\textsubscript{28K} could be important to lower [Ca\textsuperscript{2+}]\textsubscript{cyt} levels in order to maintain the necessary gradient for passive cellular Ca\textsuperscript{2+} entry and to facilitate the intracellular Ca\textsuperscript{2+} diffusional flux \cite{18}. Of interest, the Ca\textsuperscript{2+} buffer calbindin-D\textsubscript{9k} is also expressed in DCT2, but with a more focused expression than CB-D\textsubscript{28k} that extends further into the CNTs and collecting ducts (J. Loffing, personal communication).

In view of the specific expression of PV in DCT1, Belge \textit{et al.} \cite{12} investigated in detail the renal phenotype of knockout mice (KO) for PV. In comparison with wild-type littersmates, \textit{Pvalb} KO mice had increased diuresis and kaliuresis at baseline, with secondary aldosteronism and salt craving. As expected, an acute administration of the loop diuretic furosemide, aimed to increase sodium delivery in the DCT, led to increased diuresis and natriuresis/kaliuresis in both genotypes. However, surprisingly, this treatment did not increase calciuria in \textit{Pvalb} KO mice. Furthermore, \textit{Pvalb} KO mice showed no significant diuretic response to hydrochlorothiazide, but rather an accentuated hypocalciuria. The PV-deficient mice also showed an increased bone mineral density. These functional changes were explained by a strongly decreased expression of NCC at the mRNA and protein levels in the early DCT of \textit{Pvalb} KO kidneys, in the absence of any ultrastructural changes \cite{12}. The \textit{Pvalb} KO mice manifested a decreased lithium clearance, suggesting that a compensatory increase in sodium transport was taking place in the proximal tubule—as it has been reported in the case of long-term treatment with thiazide diuretics targeting NCC \cite{19}.

What could be the link between the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-buffering capacities of PV and the expression of NCC in the DCT cells? It has been known for a long time that the entire distal nephron, and DCT cells in particular, express luminal P2Y2 receptors, which trigger rapid intracellular Ca\textsuperscript{2+} transients when stimulated by purinergic agonists such as adenosine triphosphate (ATP) and
uridine triphosphate (UTP) [20]. These brief $[\text{Ca}^{2+}]_{\text{cyt}}$ transients cause a decrease in several transport systems including those involved in NaCl reabsorption [21]. Studies in a mouse DCT (mDCT) cell line showed that PV modulates the shape and duration of intracellular Ca$^{2+}$ transients induced by ATP [12]. In turn, these changes were reflected by major modifications in the expression of endogenous NCC expression in these cells. The fact that PV is capable of effectively reducing the amplitude of ATP-evoked elevations in $[\text{Ca}^{2+}]_{\text{cyt}}$ in mDCT cells [12] could be potentially linked to the regulation of NCC expression. Taken together, these studies conducted in Pvalb KO mice and in mDCT cells suggest that PV could regulate the expression of NCC by modulating intracellular Ca$^{2+}$ signalling in response to extracellular ATP in DCT cells.

Potential roles of PV in Ca$^{2+}$ and Mg$^{2+}$ handling by the kidney

Considering the buffering properties of PV, one may hypothesize that this protein could play a direct role in the transepithelial handling of Ca$^{2+}$ and Mg$^{2+}$ in the distal nephron. The major sites for transcellular Ca$^{2+}$ reabsorption in mouse kidney are the late DCT and the CNT [11]. The early part of the DCT appears to play a minor role in Ca$^{2+}$ transport as apical TRPV5 and basolateral NCX and PMCA are either not detected or only weakly expressed in this segment (Figures 1 and 2). The exclusive distribution of PV in the early DCT and the fact that its expression is independent of the vitamin D3 status also argues against a major role in distal calcium handling [13, 18]. Nevertheless, Pvalb KO mice show an increased bone mineral density and a strongly positive calcium balance when stimulated by thiazide diuretics [12]. In fact, such a phenotype is consistent with a reduced NCC expression in the DCT, similar to chronic thiazide administration. Volume contraction in the Pvalb KO mice is probably an important factor to explain the positive calcium balance, as indicated by decreased lithium clearance [12, 19]. Nonetheless, other components of the Ca$^{2+}$-signalling toolkit [3] might be modified in Pvalb KO mice and a study to address this question is underway (Schwaller, unpublished work).

It must be pointed out that even volume-repleted Pvalb KO mice tend to excrete less calcium than their wild-type littermates [12], which could in fact originate from a dysfunctional NCC. Gesek and Friedman [22] demonstrated that a reduced Cl$^{-}$ entry in DCT cells leads to decreased intracellular Cl$^{-}$ activity followed by hyperpolarization of the plasma membrane. The hyperpolarization was proposed to activate dihydroyridine-sensitive calcium channels in the apical membrane, which enhances transcellular Ca$^{2+}$ transport [22]. A similar mechanism could operate in DCT cells lacking PV, due to the down-regulation of NCC. However, it is unclear whether a hyperpolarization of the luminal membrane would persist under the chronic conditions of a life-long decrease in NCC activity. Alternatively, a decreased urinary calcium excretion may also be explained by a decreased intracellular Na$^{+}$ concentration, resulting from a decreased apical NCC activity. In this scenario, basolateral NCX1 responds by facilitating Na$^{+}$ entry into the cell and Ca$^{2+}$ exit out of the cell, enhancing the net transcellular Ca$^{2+}$ flux [23]. A third mechanism could be related to a structural hypertrophy of the TRPV5 and NCX/PMCA1b-positive CNT, as observed in NCC-deficient mice [24], which would increase the epithelial surface available for transcellular Ca$^{2+}$ reabsorption. Nevertheless, careful histological examinations have not detected such damages in the kidneys of the Pvalb KO mice. Similarly, no compensatory up-regulation of TRPV5 or calbindin-D$_{28k}$, as observed after chronic administrations of thiazide diuretics [25], has been observed in the Pvalb KO kidneys.

Elegant studies have shown that calbindin-D$_{28k}$, which is expressed in DCT2, may make use its Ca$^{2+}$-sensing properties to function differently at basal [Ca$^{2+}]_{\text{cyt}}$ and at elevated levels caused by a Ca$^{2+}$ influx. Accordingly, elevated [Ca$^{2+}]_{\text{cyt}}$ levels could induce conformational changes and regulatory processes including a negative feedback on the apical TRPV5 [17]. Nevertheless, such a mechanism seems unlikely to occur for PV, which is more a ‘simple’ Ca$^{2+}$ buffer protein than a Ca$^{2+}$ sensing and regulatory protein.

PV is distributed along with TRPM6 and NCC, in the DCT1 segment that plays a crucial role in Mg$^{2+}$ handling. The affinity of PV for Mg$^{2+}$ ($K_M$; ~30 μM) makes a suitable candidate to feasibly act as an intracellular Mg$^{2+}$ transporter/shuttle. For instance, inappropriate urinary loss of Mg$^{2+}$ is a hallmark of Gitelman syndrome (GS), an inherited tubulopathy due to loss-of-function mutations in NCC (see below). Intracellular Mg$^{2+}$ (and also Ca$^{2+}$) concentrations tightly regulate their own reuptake through a negative feedback involving apical transporters (TRPM6, TRPV5). Changes in [Mg$^{2+}]_{\text{cyt}}$ have been shown to modify regulatory elements in non-coding mRNA regions, also influencing the transcription of Mg$^{2+}$ transporters in bacteria [26]. Accordingly, a loss of PV could induce such a negative feedback (decreased apical TRPM6), which would decrease the gradient for passive cellular Mg$^{2+}$ entry and impair Mg$^{2+}$ diffusional flux [17, 27]. A mild Mg$^{2+}$ wasting phenotype has been observed in Pvalb KO mice on a C57BL/6J background (Olinger, unpublished work), but further investigations have to be done to confirm these findings.

It is important to mention that there are significant differences between Slc12a3 KO mice lacking NCC and those lacking PV. Most importantly, NCC-null mice show a more substantial and constant Mg$^{2+}$ loss in urine and hypomagnesaemia at baseline, probably consistent with severe DCT damage/loss and a loss of TRPM6-expressing cells in the distal nephron. The morphological alterations in DCT seem to be limited to its early part and are most likely a result from reduced transcellular Na$^{+}$ transport, raising the question why these ultrastructural changes were not observed in the Pvalb KO mice [12, 24]. Perhaps, the reduction in NCC activity and hence transcellular Na$^{+}$ transport in the DCTs of PV-deficient mice is not severe enough to provoke the structural changes as they occur in mice lacking any NCC activity. Experiments
addressing this issue are underway in the laboratory of Loffing.

**PVALB as candidate gene in Gitelman syndrome**

The combination of mild sodium wasting, resistance to thiazide diuretics, hypocalciuria and increased bone density observed in Pvalb KO mice is reminiscent of the manifestations of GS. GS is a recessively inherited salt-losing tubulopathy with hypokalemic alkalosis, hypomagnesaemia and hypocalciuria. The majority of patients with GS are compound heterozygous for loss-of-function mutations in the SLC12A3 gene that codes for the NCC [28]. A few cases are caused by mutations in the CLCNKB gene that codes for the basolateral ClC-Kb chloride channel in DCT cells [28, 29]. GS is probably the most common tubulopathy, with a prevalence of heterozygous carrier of an SLC12A3 mutation estimated at 1% in European populations [30]. Most patients with GS are diagnosed in adulthood and are typically normotensive, with a mild phenotype. Recent studies have pointed to the possibility of severe complications, sometimes involving children and potentially related to male gender

Despite advances in mutation detection in SLC12A3, up to 30% of patients with GS carry only a single mutant allele and negative SLC12A3 screening is observed in ∼10% of patients [29, 31]. Mutations in another gene could thus explain the lack of detection of mutant SLC12A3 alleles in GS patients. Considering the phenotype of the PV KO mouse, it was tempting to hypothesize that mutations in PVALB, the gene coding for PV, could be present in patients with GS heterozygous or in ones negative for SLC12A3 mutations [32]. The PVALB gene, localized on chromosome 22 (22q12-q13.1), has not been linked to any human disorder so far. Direct sequencing of PVALB was performed in 132 GS patients harbouring only one (n = 53) or no (n = 79) mutant SLC12A3 allele. The possible interference of biallelic SNPs (single nucleotide polymorphisms) on normal transcription or normal splicing was investigated. No sequence variants resulting in amino acid substitution or a truncated protein within the PVALB gene were found in the 264 chromosomes tested. Ten biallelic SNPs, including six novel polymorphisms, were identified: five in the 5' UTR, none of them affecting predicted regulatory elements; three in the coding region, without alteration of the consensus splice sites and two in the 3' UTR. The observed allelic frequencies did not differ significantly between GS patients and controls. These results strongly suggest that mutations in the PVALB gene are not involved in the classical form of GS [32].

Despite these negative results, it may be of interest to screen for PVALB mutations in patients harbouring neurological symptoms, such as epileptic seizures, in particular when these manifestations are linked to hypomagnesaemia. Central manifestations have been described in Pvalb KO mice, suggesting that a distinct neurological phenotype could result from the lack of PV (see below). One could also hypothesize that changes in the expression or function of PV (or in the Ca²⁺ signalling pathway in DCT cells) may participate in the individual response to thiazide diuretics [12].

**PV and renal cell carcinoma**

In more than 90% of cases, renal cell carcinoma (RCC) originates from tubular cells. The accurate typing of RCC has important implications for prognosis and therapy. In a normal clinical setting, the histologic diagnosis of RCC is made by routine light microscopy of haematoxylin-eosin stained sections. However, immunohistochemical analysis based on segmental markers is important for the differential diagnosis of non-renal cell neoplasms mimicking RCC, the differentiation of histological subtypes or rare RCC, the analysis of a small biopsy specimen and most importantly, the recognition of RCC metastases in distant organs. The immunohistochemical diagnosis of RCC is based on a set of markers indicative of the nephron segment of origin. As mentioned before, PV is exclusively expressed in the first part of the DCT in human kidney [12]. Reactivity for PV is therefore used, along with cadherin, claudins and S100A, as a highly specific and reliable marker of chromophobe RCC and benign oncocytoma, two neoplasms deriving from the distal nephron (Figure 3). In contrast, PV is not expressed in other renal neoplasms and especially in clear cell and papillary RCC.

Chromophobe carcinoma accounts for 5% of all RCC and is considered to present a rather indolent behaviour with localization restricted to the kidney and nuclear Grade 2 at presentation. It is classically divided into a typical variant and an eosinophilic variant, with a differential diagnosis including clear cell carcinoma, papillary RCC and benign oncocytoma. PV staining is a reliable marker of chromophobe carcinoma, superior to Hale’s colloidal iron and antimitochondrial 113-1 antibodies [33]. Immunostaining for other distal Ca²⁺-binding

![Fig. 3. PV as a marker of chromophobe RCC. Immunohistochemical analysis reveals a strong and ubiquitous expression of PV in a chromophobe renal carcinoma (Inset, illustrating the intense staining in chromophobe cancer cells), and staining of the early DCT of the normal renal parenchyma. (Image courtesy of Dr S. Aydin.)](http://doc.rero.ch)
proteins such as calbindin-D$_{28k}$ is most often negative and much less specific than PV for oncocytoma and chromophobe RCC. Staining for PV is of limited value to differentiate the eosinophilic variant of chromophobe RCC from oncocytoma, the most common benign renal neoplasm. The distinction is particularly challenging for so-called hybrid tumours, revealing features of both oncocytoma and chromophobe RCC and reflecting probably the common origin of these two tumours. Nevertheless, a negative or a patchy staining for PV is strongly suggestive towards the diagnosis of oncocytoma [34]. PV is also strongly expressed in the (rare) metastases from chromophobe RCC [33, 35].

Instead of being only a simple marker, could PV be a causative agent in renal tumour development? It is of interest that β-PV (oncomodulin) has been shown to activate cyclic nucleotide phosphodiesterase and can thereby act as a cellular trigger protein [36]. Oncomodulin is frequently expressed in mammalian tumours, for instance in Morris hepatoma [37]. However, PV and oncomodulin differ by their Ca$^{2+}$-binding sites. Oncomodulin has one Ca$^{2+}$-specific, non-canonical site (CD domain) and one mixed (Ca$^{2+}$/Mg$^{2+}$)-binding site (EF domain). The CD domain shows significant Ca$^{2+}$-dependent conformation changes, suggestive of additional sensor function for oncomodulin [2]. An active role of PV in tumour genesis cannot be excluded, but would be likely based on altered intracellular Ca$^{2+}$ signalling. Until now, no interacting partner indicative of a Ca$^{2+}$ sensor function has been identified.

**PV outside the kidney**

PV is highly expressed in a subgroup of inhibitory GABAergic interneurons in various brain regions including cortex, hippocampus, striatum and cerebellum (for more details, see [7]) and in fast-twitch muscle fibres [14]. It has been postulated that the absence of PV in GABAergic interneurons and in particular in chandelier cells and basket cells is correlated with an increased susceptibility to epileptic seizures in Pvalb KO mice [10].

The modulation of [Ca$^{2+}$]$_{cyt}$ kinetics in neurons lacking PV leads to increased facilitation of GABAergic transmission to postsynaptic pyramidal cells, thus resulting in a shift in the pyramidal cell’s firing properties which could, under certain experimental conditions, lead to epileptogenic insults. Indeed, the severity of pentylenetetrazole-induced generalized tonic–clonic seizures is significantly greater in Pvalb KO mice compared with wild-type littermates [10]. PV KO mice also display a mild impairment in motor coordination and motor learning [38]. There is evidence that PV plays a similar role in the epileptogenic activity of the human neocortex based on results of PV immunoreactivity in epileptic foci [39].

With respect to fast-contracting muscle activity, the contraction–relaxation cycle is prolonged in Pvalb KO muscle fibres, with a significantly greater force generated during a single twitch [9]. These changes result from an altered shape of Ca$^{2+}$ transients in the absence of PV.

A possible role of PV as a signal transduction modulator in human parathyroid gland cells by affecting [Ca$^{2+}$]$_{cyt}$ is also envisaged, potentially related to the control of PTH secretion [8].

**Conclusion and perspectives**

PV is a classical member of the EF-hand protein superfamily that plays a role in regulatory processes operating in very distinct cell types. PV has been described as a Ca$^{2+}$ buffer and Ca$^{2+}$ transporter/shuttle protein, but diverse experimental observations hint towards an additional role in magnesium handling. It is particularly puzzling that PV is exclusively expressed in the early part of the DCT of the human and mouse kidneys. A role of PV in the renal handling of electrolytes was demonstrated in Pvalb KO mice, which showed a mild salt-losing phenotype with salt craving, relatively similar to GS. A link between the Ca$^{2+}$-buffering capacity of PV and the expression of the thiazide-sensitive NCC could be established, with potential relevance for the regulation of sodium transport in the distal nephron. On the basis of these studies, PVALB has been proposed as a candidate gene in patients presenting with GS and displaying none or only a single mutant SLC12A3 allele. However, no link between mutations in PVALB and GS could be established so far. Variants in PVALB have been described, but their relevance to kidney function or response to thiazide diuretics, for instance, has not been investigated. Finally, PV is considered a reliable marker of chromophobe carcinoma and oncocytoma, two neoplasms deriving from the distal nephron. The putative role of PV in tumour genesis has not yet been investigated. The role of PV in tissues apart from the kidney is best understood in neurons, where its absence affects short-term modulation of synaptic transmission.

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