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Three functional facets of calbindin D-28k

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Many neurons of the vertebrate central nervous system (CNS) express the Ca^{2+} binding protein calbindin D-28k (CB), including important projection neurons like cerebellar Purkinje cells but also neocortical interneurons. CB has moderate cytoplasmic mobility and comprises at least four EF-hands that function in Ca^{2+} binding with rapid to intermediate kinetics and affinity. Classically it was viewed as a pure Ca^{2+} buffer important for neuronal survival. This view was extended by showing that CB is a critical determinant in the control of synaptic Ca^{2+} dynamics, presumably with strong impact on plasticity and information processing. Already 30 years ago, *in vitro* studies suggested that CB could have an additional Ca^{2+} sensor function, like its prominent acquaintance calmodulin (CaM). More recent work substantiated this hypothesis, revealing direct CB interactions with several target proteins. Different from a classical sensor, however, CB appears to interact with its targets both, in its Ca^{2+}-loaded and Ca^{2+}-free forms. Finally, CB has been shown to be involved in buffered transport of Ca^{2+}, in neurons but also in kidney. Thus, CB serves a threefold function as buffer, transporter and likely as a non-canonical sensor.

Keywords: calcium, sensor, transporter, buffer, synaptic plasticity, neurons, transmitter release

INTRODUCTION

Despite the wealth of information on expression patterns of Ca^{2+} binding proteins (CaBPs), their functional significance is only slowly emerging. In particular, this is due to their complex interplay with other Ca^{2+} controlling mechanisms and the inherent technical difficulties in studying biophysical properties of individual proteins (Neher, 2000), including the differentiation between Ca^{2+}-buffer and Ca^{2+}-sensor (da Silva and Reinach, 1991). Buffers are characterized by more or less specific binding/chelating of Ca^{2+} ions without further Ca^{2+}-dependent target interactions. Their function is in the control of the spatiotemporal extent of Ca^{2+} signaling domains (Augustine et al., 2003; Eggermann et al., 2012). Sensors, on the other hand, undergo additional characteristic conformational changes upon Ca^{2+}-binding, resulting in exposure of hydrophobic surfaces necessary for binding and subsequent regulation of downstream effectors (Ikura, 1996; Schwaller, 2008, 2010). Their functional significance lies in both, the control of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and in triggering Ca^{2+}-dependent downstream signaling.

In consequence, characterization of a CaBP requires determination of several biophysical parameters (**Table 1**), including affinity and kinetics of Ca^{2+}-binding, intracellular mobility, structural and conformational analysis, and the identification of binding partners. Following some general remarks on buffering, I will review advances in gathering biophysical parameters of CB that allowed deducing its functional facets, with emphasis on its neuronal function.

SOME GENERAL ASPECTS OF BUFFERING

Dissociation constants (K_D) of proton buffers are optimized to clamp pH at 7–7.4 in living tissue by bidirectional buffering of free protons to a concentration of ~100 nM. $[Ca^{2+}]_i$ in resting cells is similar, however, contrasting to pH buffers, K_D values of most CaBPs are well above $[Ca^{2+}]_i$; a notable exception is parvalbumin (PV, $K_{D,Ca}$ ~9 nM; Lee et al., 2000b). Thus, under resting conditions most binding sites are unoccupied by Ca^{2+}, such that CaBPs limit increases in $[Ca^{2+}]_i$ from the resting level rather than clamping $[Ca^{2+}]_i$ at a given level, i.e., they act as unidirectional buffers. This can even be augmented by an additional Mg^{2+} affinity, which reduces the effective affinity for Ca^{2+} due to competition or a necessity for preceding Mg^{2+} unbinding (**Figure 1**).

Neuronal Ca^{2+} signals are typically short lived and the amount of Ca^{2+} bound to a specific buffer can substantially deviate from the steady state value (Markram et al., 1998). Non-equilibrium conditions require a kinetic description of Ca^{2+} binding by forward (k_{on}) and backward (k_{off}) rates, values that could be quantified only recently due to a notable technical advance (Nägerl et al., 2000; Faas et al., 2007, 2011).

I will close these general remarks with a note on the ambiguous term "saturation." In biochemistry, saturation refers to the fraction of total binding sites occupied at a given time. In chemistry, it often marks the 100% occupancy, probably the most intuitive meaning. In descriptions of Ca^{2+} dynamics saturation is often used to mark the deviation from linearity. A linear signaling process has the property that the combined effects of two or more elementary events result in a response which is given by the sum of the individual responses. This is the case if increases in $[Ca^{2+}]_i$ are much smaller than K_D of the buffer(s). Ca^{2+} kinetics become more complex if the increase in $[Ca^{2+}]_i$ approaches K_D (Neher, 1998a), i.e., saturation of a CaBP occurs around its half-occupancy.

Ca^{2+} BINDING AND MOBILITY

CB has six EF-hands, of which one or two are non-functioning in metal binding (Leathers et al., 1990; Åkerfeldt et al., 1996;

Table 1 | Properties of calbindin D-28k.

Parameter[a]	Value	References/Notes
Amino acids	260–261	Celio et al., 1996, species specific
Molecular weight	29–30 kDa	Celio et al., 1996
Diffusion coefficients in		
Water	$>100\,\mu m^2 s^{-1}$	Gabso et al., 1997
Spiny dendrites of Purkinje neurons	$20 \pm 2\,\mu m^2 s^{-1}$	Schmidt et al., 2005*
Intracellular concentration in		
Purkinje neurons	100 to $<360\,\mu M$	Kosaka et al., 1993; Maeda et al., 1999
Somata of Purkinje neurons	$208 \pm 42\,\mu M$	Hackney et al., 2005
Hippocampal granule cells	$0–40\,\mu M$	Müller et al., 2005[a]
CA1 pyramidal neurons	$45 \pm 2\,\mu M$	Müller et al., 2005
CA3 interneurons	$47 \pm 6\,\mu M$	Müller et al., 2005
Rat inner hair cells	$40–125\,\mu M$	Hackney et al., 2005[b]
Rat outer hair cells	$57–197\,\mu M$	Hackney et al., 2005[b]
Binding sites		Leathers et al., 1990; Åkerfeldt et al., 1996; Berggård et al., 2002a[c]
Total	6	
Functional	4	Mixed Ca^{2+}/Mg^{2+} binding sites; the remaining 2 EF hands do not bind Ca^{2+} (EF-2) or with a very low affinity (EF-6)
Metal binding		
Mg^{2+} $K_{D,Mg}$	$714\,\mu M$	Berggård et al., 2002a
Ca^{2+} $K_{D,Ca}$	393 nM	Faas et al., 2011
k_{on}	$75\,\mu M^{-1}s^{-1}$	Faas et al., 2011
k_{off}	$29.5\,s^{-1}$	Faas et al., 2011
Cooperativity		
n_H	1.2–1.3	presumably Mg^{2+} dependent[d]
Binding to *myo*-inositol monophosphatase		Berggård et al., 2002b
K_D	$0.9\,\mu M$	Berggård et al., 2002b
k_{off}	0.08 (0.06–0.1)[#] s^{-1}	Schmidt et al., 2005, Purkinje neurons

** Errors as SEM, unless stated otherwise.*

[a] Developmentally-regulated differences.

[b] Field of view averages, disregarding strong developmental differences and differences between apical and basal cells.

[c] Unless otherwise noted all following parameters were obtained from cuvette measurements.

[d] Hill coefficient was estimated by Faas et al. (2011) based on data from Berggård et al. (2002a).

[#] Interquartile range.

Berggård et al., 2002a; Cedervall et al., 2005; Kojetin et al., 2006). EF-2 is consistently viewed as non-metal binding while EF-6 may have a very low Ca^{2+} affinity (Åkerfeldt et al., 1996; Cedervall et al., 2005). EF-1, 3, 4, 5 are mixed metal binding sites, however, with much higher affinity for Ca^{2+} (393 nM; Faas et al., 2011) than for Mg^{2+} (714 μM; Berggård et al., 2002a). Thus, under physiological conditions ($[Mg^{2+}]_i$ ~600 μM) CB will function predominantly as a Ca^{2+} buffer. In resting neurons ($[Ca^{2+}]_i$ ~50 nM, $[Mg^{2+}]_i$ ~600 μM), it will be loaded by approx. ten percent with Ca^{2+} and 45% with Mg^{2+} (**Figure 1**; Berggård et al., 2002a). Compared to the related PV, the presence of Mg^{2+} produces only a minor shift in the apparent $K_{D,Ca}$ of CB (**Figure 1**), while it appears to increase cooperativity in Ca^{2+} binding (Berggård et al., 2002a); however, cooperativity is still negligible, with an estimated Hill coefficient (n_H) of ~1.25 (Faas et al., 2011).

Disregarding cooperativity, CB binds Ca^{2+} with rapid to intermediate kinetics (k_{on} $75\,\mu M^{-1}s^{-1}$) and medium (393 nM) affinity (Faas et al., 2011; **Table 1**). Its on-rates are between those of EGTA (k_{on} $10\,\mu M^{-1}s^{-1}$, K_D 70 nM; Nägerl et al., 2000; Meinrenken et al., 2002) and BAPTA (k_{on} $400\,\mu M^{-1}s^{-1}$, K_D 220 nM; Naraghi, 1997; Naraghi and Neher, 1997; Meinrenken et al., 2002), while its affinity is closer to BAPTA. Typically, these properties endow CB to function as a major determinant of neuronal Ca^{2+} kinetics (Airaksinen et al., 1997; Barski et al., 2003; Schmidt et al., 2003).

The diffusion of CB has been quantified in spiny dendrites of cerebellar Purkinje neurons (PNs). Apart from a smaller immobilized fraction (see below), CB diffused with an apparent diffusion coefficient (D) of $20\,\mu m^2/s$ between spines and parent dendrites (Schmidt et al., 2005). This is ~2-fold slower than the diffusional mobility of PV in the same cellular compartments, but identical to the D of a mobile calmodulin (CaM) fraction in HEK293 cells (Kim et al., 2004) and in spiny dendrites of PNs (Schmidt et al., 2007). To my knowledge there is no quantification of aqueous CB

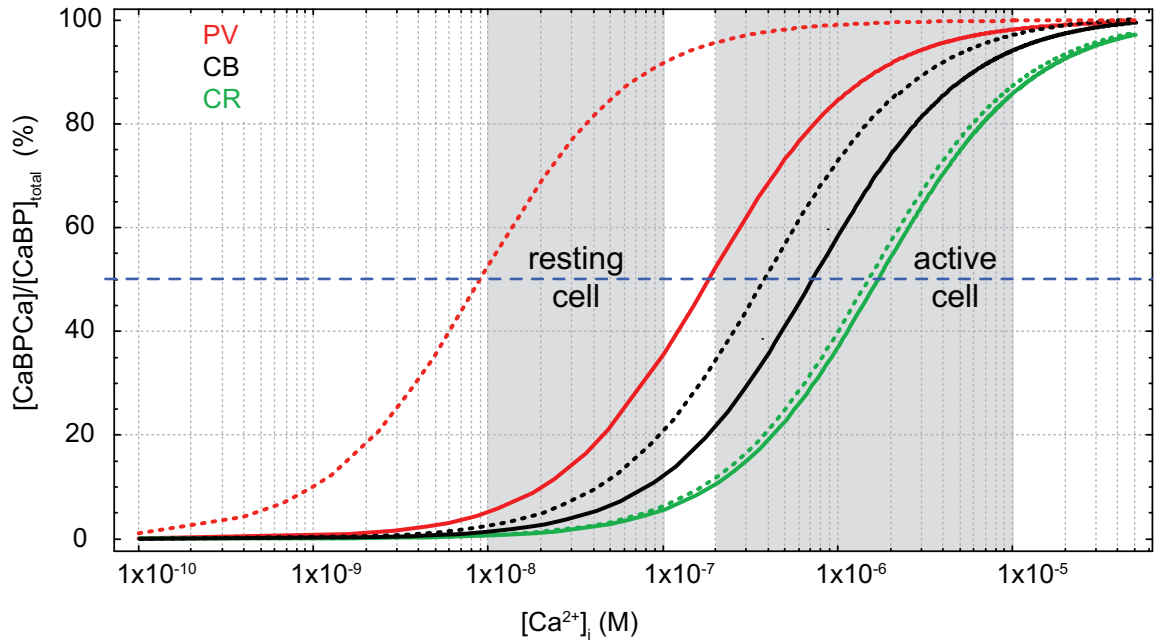

FIGURE 1 | Effects of Mg^{2+} on Ca^{2+} binding. Simulated steady-state Ca^{2+}-binding curves of the Ca^{2+} binding proteins (CaBP) parvalbumin (PV, red, $K_{D,Ca}$ 9 nM, $K_{D,Mg}$ 31 μM), calbindin (CB, black, average $K_{D,Ca}$ 393 nM, $K_{D,Mg}$ 714 μM; cf. **Table 1**), and calretinin (CR, green, average $K_{D,Ca}$ 1.5 μM, $K_{D,Mg}$ 4.5 mM) in the absence of Mg^{2+} (dotted lines) and in the presence of 600 μM Mg^{2+} (solid lines).

diffusion, but it might be estimated to be $>100\,\mu m^2/s$ (Gabso et al., 1997).

BUFFER OR SENSOR?

In vitro studies from the 80th and 90th already suggested that CB might have an additional Ca^{2+} sensor function. It was shown to activate isolated erythrocyte membrane Ca^{2+}-Mg^{2+} -ATPases (Morgan et al., 1986) and cyclic nucleotide phosphodiesterases (Reisner et al., 1992); in centrifugation studies of different tissues CB was found not only in the cytoplasmatic fractions but also in membrane/organelle containing fractions (Hubbard and McHugh, 1995; Winsky and Kuźnicki, 1995). The CB content in the membrane fractions was decreased in samples prepared or incubated in low Ca^{2+} (Winsky and Kuźnicki, 1995).

Three studies from the group of S. Linse (Berggård et al., 2000, 2002a,b) laid the foundation for substantiating the sensor hypothesis: They found that CB underwent substantial conformational rearrangements upon Ca^{2+}-binding and protonation, likely exposing EF-2, but not upon Mg^{2+}-binding. These changes went beyond the moderate redistributions in Mg^{2+}-induced cooperativity in Ca^{2+}-binding (see above). Unlike classical sensors CB had exposed hydrophobic regions also in its Ca^{2+}-free (apo-) conformation, which is thermodynamically unfavorable under aqueous conditions, thus, suggesting additional Ca^{2+}-independent interactions with target proteins. Subsequently, CB was shown to interact with *myo*-inositol monophosphatase-1 (IMPase), a key enzyme in the IP3 second messenger pathway (see below) both, in its apo- and Ca^{2+}-bound form with low affinity (K_D ~0.9 μM). Finally, they identified a 12 amino-acid motive as the putative CB binding domain of IMPase.

Another CB ligand identified is Ran-binding-protein-M (RanBPM), a small GTPase involved in nuclear transport processes and microtubule formation (Lutz et al., 2003). Using NMR, it was shown that RanBPM interacted with Ca^{2+} loaded CB. Finally, CB was found to inhibit caspase-3 in osteoblastic cells independent of Ca^{2+}, thereby, suppressing apoptosis (Bellido et al., 2000). Caspases are important enzymes in apoptosis, with activation of caspase-3 triggering the common executive pathway of cell death (Grütter, 2000; Yuan and Yankner, 2000; Yan and Shi, 2005). In central nervous system (CNS), incorrect execution of death pathways is thought to be associated with severe disorders including Chorea Huntington and Alzheimer disease. The finding that CB markedly reduces caspase-3 activity is particularly noteworthy in this context, since it might act as a neuroprotective agent.

So far, only *in vitro* studies showed a sensor function of CB. The CB-IMPase interaction was demonstrated *in situ* in PNs in acute slices (Schmidt et al., 2005). In multi-photon fluorescence recovery after photobleaching experiments a fraction of dye-labeled CB (~20–30%) was found to be immobilized for >1 s in spines and dendrites, but not in smooth parts of axons. Use of the above peptide sequence from IMPase in a competition assay led to significant relief from immobilization, suggesting that CB indeed interacted with IMPase in PNs. Further experiments showed that the interaction was influenced by synaptic activation associated with increased $[Ca^{2+}]_i$.

A detailed NMR analysis of CB structure (Kojetin et al., 2006), completing earlier work (Klaus et al., 1999; Berggård et al., 2000, 2002a,b; Venters et al., 2003; Venyaminov et al., 2004; Vanbelle et al., 2005), confirmed that upon Ca^{2+} binding CB adopts

discrete hydrophobic states but also has exposed hydrophobic surfaces in its apo-form. In addition, the regions mediating the interactions with RanBPM, IMPase, Caspase-3 and also its pro-domain were mapped.

Finally, in kidney CB was found to associate with TRPV5 channels which are involved in Ca^{2+} reabsorption from the urea (Lambers et al., 2006). The family of TRP channels is extraordinarily large with several members being abundantly expressed in the CNS. While it is temping to speculate on further direct CB interactions with Ca^{2+}-conductances in neurons, experimental evidence is missing.

Taken together, there is growing evidence that CB not only functions as Ca^{2+}-buffer but also binds to and regulates a variety of target proteins, including membrane ATPases, IMPase, RanBPM, procaspase-3, caspase-3, and TRPV5. Unlike canonical Ca^{2+}-sensors, however, CB likely interacts in Ca^{2+}-free and Ca^{2+}-occupied form with its targets.

FUNCTIONAL ASPECTS OF CB

CB appears to fulfill three functions: First, it functions as a mobile or partly immobilized Ca^{2+}-buffer with medium kinetics and affinity—buffer function. Second, it functions in buffered Ca^{2+}-diffusion—transport function. Finally, it interacts with target proteins likely both, in its apo- and Ca^{2+}-loaded form—sensor-like function.

BUFFER FUNCTION

In neurons expressing CB, it makes a major contribution to the total buffer capacitance (Fierro and Llano, 1996; Jackson and Redman, 2003; but see Faas et al. (2011) for an alternative view on CA1 spines in the presumed presence of large amounts of CaM). In dendrites and spines, CB clips the peak amplitude of synaptically induced Ca^{2+} transients, speeds their initial decay kinetics and prolongs their later phase (Airaksinen et al., 1997; Schmidt et al., 2003, 2007), while the rise time of the Ca^{2+} transients remained essentially unaffected (Koster et al., 1995; Schmidt et al., 2003). Thus, in postsynaptic structures CB induces characteristic biphasic decay kinetics of volume averaged Ca^{2+} transients and controls their amplitude.

Long lasting alterations of synaptic weight, like long-term potentiation (LTP) or depression (LTD) comprise strong Ca^{2+}-dependent postsynaptic components. LTP in CA1 pyramidal neurons with reduced CB content could be induced normally but its maintenance was affected, leading to impaired spatial learning (Molinari et al., 1996). Whether this is attributable to altered Ca^{2+}-signaling in the absence of CB or a direct target modulation by CB remained unclear. Lack of CB results also in deficits in motor coordination (Airaksinen et al., 1997; Barski et al., 2003), which is consistent with the strong CB expression in cerebellar cortex and its impact on synaptically mediated Ca^{2+} transients. However, parallel-fiber (PF) LTD in PNs lacking CB was normal and also Ca^{2+}-signals mediated via activation of metabotropic glutamate receptors (mGluRs), known to be required in LTD induction (Daniel et al., 1998; Ito, 2001), were unaltered compared to the WT (Barski et al., 2003). It remained elusive, however, why rapid Ca^{2+} transients mediated by climbing-fiber and PF inputs were affected by lack of CB, whereas longer lasting, mGluR mediated Ca^{2+}-signals were not (cf. discussion in Barski et al., 2003 for a possible explanation). Given that the conditional knock-outs used in the study are not affected by compensations for lack of CB (Vecellio et al., 2000; Kreiner et al., 2010), the answer may involve the as yet not further characterized CB-IMPase interaction (Schmidt et al., 2005; cf. below).

In presynaptic terminals, relevant Ca^{2+}-signaling domains and their topographical relationships to Ca^{2+} dependent processes are well defined (Neher, 1998b; Augustine et al., 2003; Eggermann et al., 2012). However, the function of individual CaBPs has been rarely specified, although they are generally believed to be crucial in regulating transmitter release and short-term plasticity. Specifically, it has been postulated that saturation of CB underlies a form of paired pulse facilitation (PPF) at neocortical interneuron to pyramidal neuron synapses, hippocampal mossy-fiber to CA-3 synapses (Blatow et al., 2003), and recurrent PN synapses (Orduz and Llano, 2007). This form of PPF has been termed "pseudofacilitation" (Rozov et al., 2001) in order to distinguish it from more classical mechanisms like residual Ca^{2+} (Zucker and Stockbridge, 1983; Connor et al., 1986), Ca^{2+} remaining bound to the release sensor or a facilitation sensor ("active Ca^{2+}"; Katz and Miledi, 1968; Yamada and Zucker, 1992; Atluri and Regehr, 1996) or Ca^{2+} dependent facilitation of Ca^{2+}-currents (CDF; Lee et al., 1999, 2000a; Tsujimoto et al., 2002; for more detail see, e.g., Neher, 1998b; Zucker and Regehr, 2002; Stevens, 2003). In pseudofacilitation a substantial amount of Ca^{2+} entering the presynapse during the first action potential (AP) is thought to be buffered by CB, thereby, reducing the initial release probability but also saturating CB, which in turn results in increased $[Ca^{2+}]_i$ during the second AP and potentiated release (Neher, 1998b; Rozov et al., 2001; Blatow et al., 2003; Felmy et al., 2003). Albeit direct evidence for CB saturation is scarce and Ca^{2+} imaging experiments from CB containing versus CB deficient presynaptic terminals are lacking, the hypothesis is consistent with conclusions drawn from experiments with BAPTA (Rozov et al., 2001). However, Ca^{2+} binding by endogenous CaBPs can be more complex than binding by exogenous buffers, as exemplified recently for PV (Caillard et al., 2000; Eggermann et al., 2012; Eggermann and Jonas, 2012). Thus, more direct evidence for saturation is desirable.

CDF and its counterpart Ca^{2+}-dependent inactivation (CDI) are Ca^{2+} driven feedback mechanisms regulating voltage operated $Ca_v2.1$ (P/Q type) Ca^{2+}-channels. CDF is mediated by Ca^{2+} loaded CaM or NCS-1 (Lee et al., 1999, 2000a; Tsujimoto et al., 2002), while CB only affected CDI but not CDF. Different from the CaM and NCS-1 effects on CDF, CB effects on CDI were essentially consistent with its buffering action and did not require the assumption of a Ca^{2+}-sensor function (Kreiner and Lee, 2006).

TRANSPORT FUNCTION

Beyond Ca^{2+} transporting epithelia (Bronner and Stein, 1988; Bronner, 1989; Koster et al., 1995; Lambers et al., 2006), buffered Ca^{2+} transport by CB has recently also been suggested between activated spines and their parent dendritic shafts (Schmidt et al., 2007; Schmidt and Eilers, 2009). Although spill-over of Ca^{2+} from spines into dendrites had been reported before by several

groups (Majewska et al., 2000; Holthoff et al., 2002; Schmidt et al., 2003), these experimental observations were significantly influenced by the action of Ca^{2+} dyes (Sabatini et al., 2002; Schmidt et al., 2003) and in the majority view spine necks remained substantial diffusion barriers for second messengers, making spines biochemically isolated compartments (Gamble and Koch, 1987; Zador et al., 1990; Müller and Connor, 1991; Svoboda et al., 1996; Sabatini et al., 2002). An analysis of spine Ca^{2+} dynamics under minimally perturbed conditions, however, confirmed that indeed most spines of pyramidal neurons allow a sizeable Ca^{2+} efflux that was tightly regulated by the geometry of the spine neck (Noguchi et al., 2005). Consecutively, it was shown that in PNs the majority of Ca^{2+} left the spine bound to CB (Schmidt et al., 2007), with the buffered efflux again being tightly controlled by the spine neck geometry (Schmidt and Eilers, 2009). This diffusional coupling drove a spatial summation process in which coincident activity of neighboring spines was integrated in the dendrite with the potential to activate dendritic CaM. This biochemical summation might exist in parallel to the classical summation of electrical signals in dendrites, possibly with reciprocal interaction (Nemri and Ghisovan, 2007).

Despite buffered Ca^{2+} transport, even neurons expressing large amounts of CB can retain Ca^{2+} signals that are spatially restricted to activated dendritic branches (Eilers et al., 1995, 1997) Although significant Ca^{2+} transport out of the active branches indeed occurred, it was outweighed by Ca^{2+} extrusion along the dendrite. This close interplay between diffusion and extrusion defined the capability of Ca^{2+} to spread between dendritic branches (Schmidt et al., 2011).

SENSOR-LIKE FUNCTION

In this final function-section, I will focus on the neuronal CB-IMPase interaction. IMPase catalyzes the hydrolysis of *myo*-inositol-1(or 4)-monophosphate to form free *myo*-inositol, the resource for IP3 and DAG second messengers. CB was shown to bind IMPase *in vitro* (Berggård et al., 2002b) and in PNs (Schmidt et al., 2005) with a K_D of $\sim$0.9 μM and an off-rate of $\sim$0.08 s^{-1} (**Table 1**). *In vitro*, apo- and Ca^{2+}-bound CB activated IMPase similarity up to 250-fold. The activation was most pronounced under conditions that otherwise were associated with very low IMPase activity, precisely at reduced pH and at low substrate concentration. In spiny dendrites, binding of CB to IMPase was apparent at resting Ca^{2+} levels but further amplified by synaptic activation associated with increases in intracellular Ca^{2+} in a frequency dependent way. Thus, while the *in vitro* interaction appeared essentially independent of Ca^{2+}, in dendrites it is likely boosted by increasing Ca^{2+} levels.

Still unresolved is the impact of the CB-IMPase interaction on dendritic IP3 mediated Ca^{2+}-signaling. Two scenarios would be conceivable: First, the increased IMPase activity speeds the degradation of IP and could, in consequence, result in accelerated IP3 degradation and in reduced Ca^{2+}-signals. Second, increased IMPase activity could result in an accelerated source substance supply for IP3 production and consequently in increased IP3 levels and amplified Ca^{2+}-signaling. Considering that IP3 mediated Ca^{2+}-signals in dendrites of PN-specific CB knock-outs were, despite the absence of a major buffer, unaltered compared to the WT (Barski et al., 2003) this argues in favor of the second scenario.

CONCLUDING REMARKS

I reviewed evidence for a threefold function of CB, consisting of a sensor-like and a transport function in addition to buffering of Ca^{2+}. Different from a canonical Ca^{2+}-sensor, CB appears to bind its targets in Ca^{2+}-occupied and Ca^{2+}-free conformation. Spino-dendritic Ca^{2+}-coupling and its regulation by the geometry of the spine neck were shown for pyramidal as well as PNs. In the latter the coupling was essentially mediated by CB. The control of this coupling via the spine neck, which itself undergoes use-dependent regulation, will increase the computational capacitance of dendrites. Despite growing evidence for these two additional functions, decades of investigation on CB mainly underlined its importance as a Ca^{2+}-buffer.

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