

Two distinct calcium-calmodulin interactions with N-terminal regions of the olfactory and rod cyclic nucleotide-gated channels characterized by NMR spectroscopy

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Abstract The interactions of calcium-calmodulin with two fragments of the N-terminal domains of the olfactory α -subunit and rod β -subunit cyclic nucleotide-gated channels have been investigated using nuclear magnetic resonance spectroscopy. The results indicate that in the two cases both the N-terminal and the C-terminal calmodulin lobes are involved in the interaction. The olfactory cyclic nucleotide-gated channel segment forms a 1:1 complex with calmodulin, whereas the rod fragment forms a 2:1 complex. The correlation times of the two complexes, as estimated by ^{15}N relaxation studies, are compatible with the observed stoichiometries. These results indicate differences in the mode of action by which calmodulin modulates the activity of both channels, and suggest either that the rod channel is modulated through a simultaneous interaction of two β -subunits with calmodulin or that other regions of the N-terminus are necessarily implicated in the binding.

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1. Introduction

Ion channels are membrane proteins that play a fundamental role in cell physiology and in signal transduction and transmission. Since the discovery in the mid-1980s that the cation channel mediating visual transduction in retinal rod photoreceptors was directly activated by cGMP [1,2], a number of ion channels with the same property have been reported that are involved in multiple cell functions. These channels, classified as cyclic nucleotide-gated (CNG) channels (for a review see [3]), are hetero-oligomers, and are composed of different subunits. Each subunit comprises a N-terminal domain, a transmembrane domain formed by six helices and

a pore region, and a C-terminus where the cyclic nucleotide binding domain is located [3].

The activity of both rod and olfactory CNG channels is modulated by Ca^{2+} -calmodulin (CaM) by decreasing the apparent affinity for the cyclic nucleotide upon binding to the channel [4]. The binding region of CaM has been identified in the α -subunit N-terminal domain in the olfactory CNG channel [5] and in the β -subunit N-terminal domain in the rod CNG channel [6,7]. For both channels, CaM binding to an N-terminal region of the channel produces a disruption of an interaction between this region and a C-terminal region, causing inhibition [8,9]. However, the precise molecular mechanisms of inhibition are different. Binding of CaM produces a 10-fold affinity reduction for the cyclic nucleotide in the olfactory CNG channel, and only a two-fold decrease in the rod channel [5–8]. Deletion of the binding site for CaM decreases the affinity for the cyclic nucleotide in the olfactory CNG channel, but not in the rod channel [5,8,9]. Finally, the interdomain interaction involves different subunits and regions [8,10].

A full understanding at a molecular level of the CaM regulatory mechanism of olfactory and rod CNG channels requires the structure elucidation of complexes between CaM and the N-terminal domains, and those formed by the intramolecular interaction of N- and C-terminal domains. No structural information is yet available regarding these complexes. As a first step in this direction, in the present article we used nuclear magnetic resonance (NMR) spectroscopy to perform a comparative study of the interaction of ^{15}N - and $^{15}\text{N}/^{13}\text{C}$ -labeled CaM with two synthetic peptides, bOCNCp, corresponding to residues 62–87 (QQRGGFRRIARLVGVLEWAYRNFR) of the olfactory CNG channel α -subunit, and bRCNCp, corresponding to residues 676–701 (AIINDRLQELVKLFKERTEKVKEKLI) of the rod CNG channel β -subunit.

2. Materials and methods

Uniformly ^{15}N - or $^{15}\text{N}/^{13}\text{C}$ -labeled recombinant *Xenopus laevis* CaM (identical to mammalian CaM) was expressed in *Escherichia*

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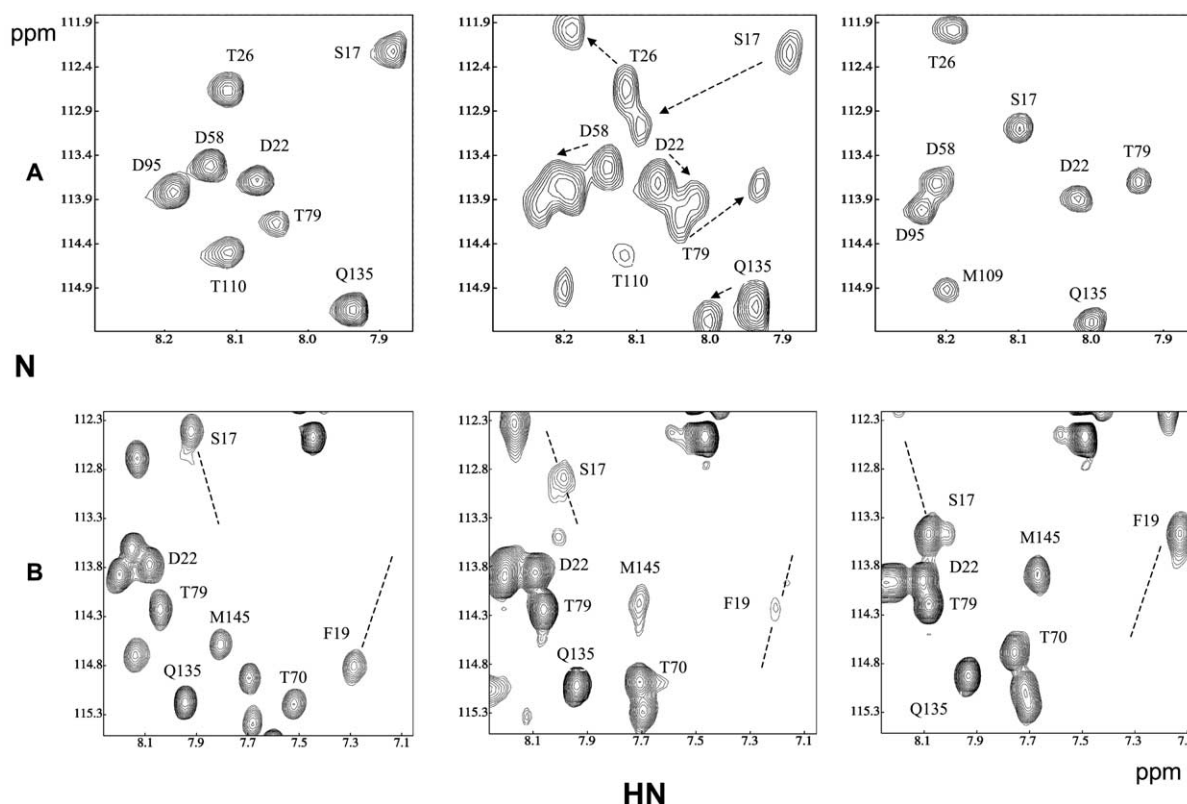


Fig. 1. Selected regions of the ^1H , ^{15}N HSQC of ^{15}N -labelled CaM interacting with bOCNCp (A) and bRCNCp (B). Spectra from left to right of each row were obtained by addition of the following equivalents of the peptides: 0, 0.5, 1.0 eq for A, and 0, 1.0, 2.0 eq for B.

coli strain AR58 and the purification was carried out using a phenyl Sepharose column using a slight modification of the previously reported procedure [11]. bOCNCp and bRCNCp were purchased from Epytop (France).

NMR samples contained 0.4–1.2 mM complex in CaCl_2 6 mM, pH 6.5. All NMR spectra were recorded at 35°C on a Bruker Avance 700 MHz spectrometer equipped with pulsed field gradient triple-resonance probes. Spectra were processed using the NMRPipe software package [12] and analyzed with NMRView [13]. Complex formation was followed by titration of CaM with increasing concentrations of the peptides. Assignments of the ^1H , ^{15}N , ^{13}CO and ^{13}C resonances of bOCNCp were based on the following experiments: CBCA(CO)NH, HBHA(CO)NH, CC(CO)NH-TOCSY, HNC0, HNCA, HN(CO)CA, 3D ^{15}N -edited TOCSY-HSQC, HCCH-TOCSY, HCCH-COSY, HACACO, N,C-filtered TOCSY and NOESY. Chemical shifts were confirmed by inspection of intraresidue and sequential NOEs in the ^1H , ^{15}N -NOESY-HSQC and ^1H , ^{13}C -NOESY-HMQC experiments. Assignments of the ^1H , ^{15}N and ^{13}CO resonances of the bRCNCp complex were performed by following the shifts of free CaM NHs upon addition of the peptide, and confirmed both by the use of a HNC0 experiment and a ^1H , ^{15}N -NOESY-HSQC and by comparison with previously reported chemical shifts of both free CaM [14], and Ca^{2+} /CaM-peptide complexes already reported [15–19].

^{15}N relaxation constant T_2 were measured in a full interleaved manner using a standard sequence [20]. Six delays were used for T_2 measurements of 8.2, 24.5, 40.8, 57.1, 72.4 and 97.9 ms. Data were fitted using the Rate Analysis routine of NMRView [13].

3. Results

The interaction of CaM with bOCNCp and bRCNCp has been followed by recording ^1H , ^{15}N HSQC spectra upon increasing concentrations of bOCNCp and bRCNCp. Fig. 1 shows two selected regions of the ^1H , ^{15}N HSQC. After addition of 1 equivalent of bOCNCp no signals belonging to free

CaM are detectable, indicating the formation of a 1:1 complex. Sequential assignment of bOCNCp–CaM complex was completed using an array of triple-resonance experiments on samples of ^{15}N - or $^{15}\text{N}/^{13}\text{C}$ -labeled CaM complexed with unlabelled bOCNCp as described in Section 2. The ^1H , ^{13}C and ^{15}N chemical shifts concerning the CaM and the peptide in the CaM–bOCNCp complex have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under BMRB accession number 5480.

Titration with bRCNCp reveals that only after addition of 2 equivalents all measurable changes in the position of the peaks reach the maximum effect. No further shifts are detected upon addition of a third equivalent. The effects on peak positions are linearly dependent on the increasing concentration of the peptide until a 2:1 ratio is reached. This observation indicates a 2:1 complex formation and favors a model of simultaneous binding of the two peptides to CaM.

The data reported in Fig. 1 indicate that the exchange kinetics of the two complexes described, following the bound and free CaM signals, are very different. In the case of bOCNCp, two set of signals can be readily distinguished (Fig. 1A, central panel) corresponding to bound and free CaM, indicative of a low rate constant (k_{off}) for the release of the ligand [21]. The interaction with bRCNCp leads to an exchange kinetics in fast to intermediate exchange between free and bound CaM. Line-broadening effects are clearly visible for some peaks like S-17 and F-19 in Fig. 1B. As a consequence of the distinct exchange kinetics observed, the lifetimes of the two complexes are very different. A quantitative analysis of our data leads to the estimation of lifetimes longer than 2 s and around 1 ms for CaM–bOCNCp and CaM–

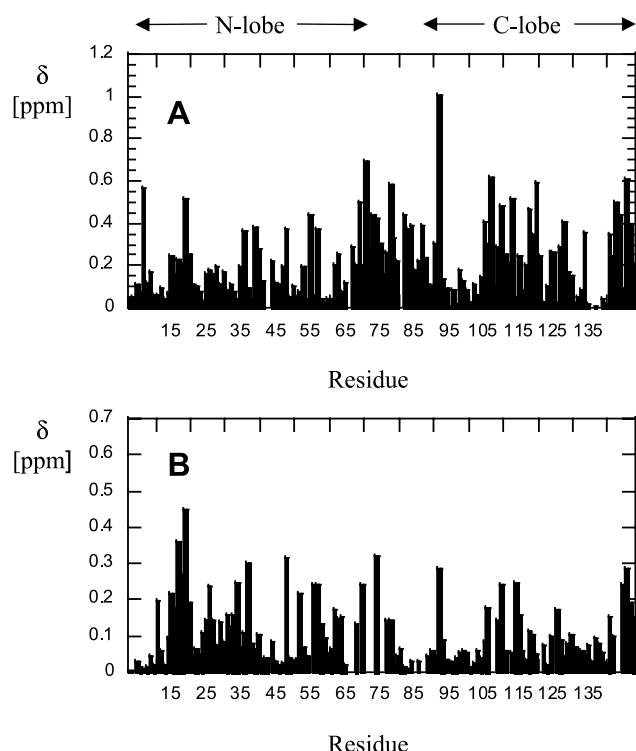


Fig. 2. Chemical shift difference for the whole HN group between free and bound CaM complexed with bOCNCp (A) and bRCNCp (B). Chemical shifts for free CaM were taken from Ikura et al. [14]. A single quantity was used to describe shifts of both nuclei in the HN group: $\delta = \sqrt{0.5(\Delta\delta_H^2 + \Delta\delta_N^2/25)}$ [32]. Some residues in the central linker of the complex with bRCNCp were not assigned due to severe overlap.

bRCNCp respectively. This result partially reflects the differences in affinities established for similar peptides ($K_d = 4\text{--}12$ nM for the rat counterpart of bOCNCp [5], and $K_d = 0.2\text{--}0.5$ μM for bRCNCp [6]).

Fig. 2 shows the chemical shift differences for the NH groups caused by the interaction of CaM with bOCNCp and bRCNCp respectively. CaM is a protein formed by two lobes, each comprising two Ca^{2+} binding sites, connected by a central linker. Each lobe presents a series of pockets that interact with hydrophobic residues of the target [22]. Interaction with the two peptides exerts an effect on the chemical shift of NHs in both the N- and the C-lobe, implying that both regions of CaM are involved in the interactions. The same conclusion can be reached following the chemical shift changes of the ϵ -methyl groups of methionines during the titration (Fig. 3) using a 2D version of the methionine-edited $^1\text{H}\epsilon\text{-}^{13}\text{C}\epsilon$ spectrum [23] that allows the selective detection of the methyl groups of methionines. Upon interaction with bOCNCp, all nine CaM methionines change their chemical shifts, showing a slow exchange kinetics as in the titration of Fig. 1. Titration of CaM with bRCNCp produces a large broadening of the ϵ -methyl signals for methionines belonging to both the N- and C-lobe. After addition of 2 equivalents of bRCNCp, new signals with a large chemical shift dispersion are observable. This result implies that both the N- and the C-lobe of CaM are involved in the interaction with bRCNCp. Moreover, the ϵ -methyl signals of most methionine residues are visible only after addition of the second equivalent of bRCNCp. This result is further evidence of the simultaneous binding of two peptide molecules to CaM.

We then investigated the effect of bOCNCp and bRCNCp complexation on the molecular correlation time, τ_c , by measuring the T_2 relaxation rates of ^{15}N backbone amides for residues located in the helices of CaM. Selected curves are presented in Fig. 4A,B, and average values for helices of both lobes, considered separately and together, are presented in Table 1. Residues located in the eight helices of CaM present a reduced local mobility showing a very uniform and high global order parameter, S^2 [20]. There is compelling evidence that binding of different peptides to CaM does not change the helix secondary structure independently of the

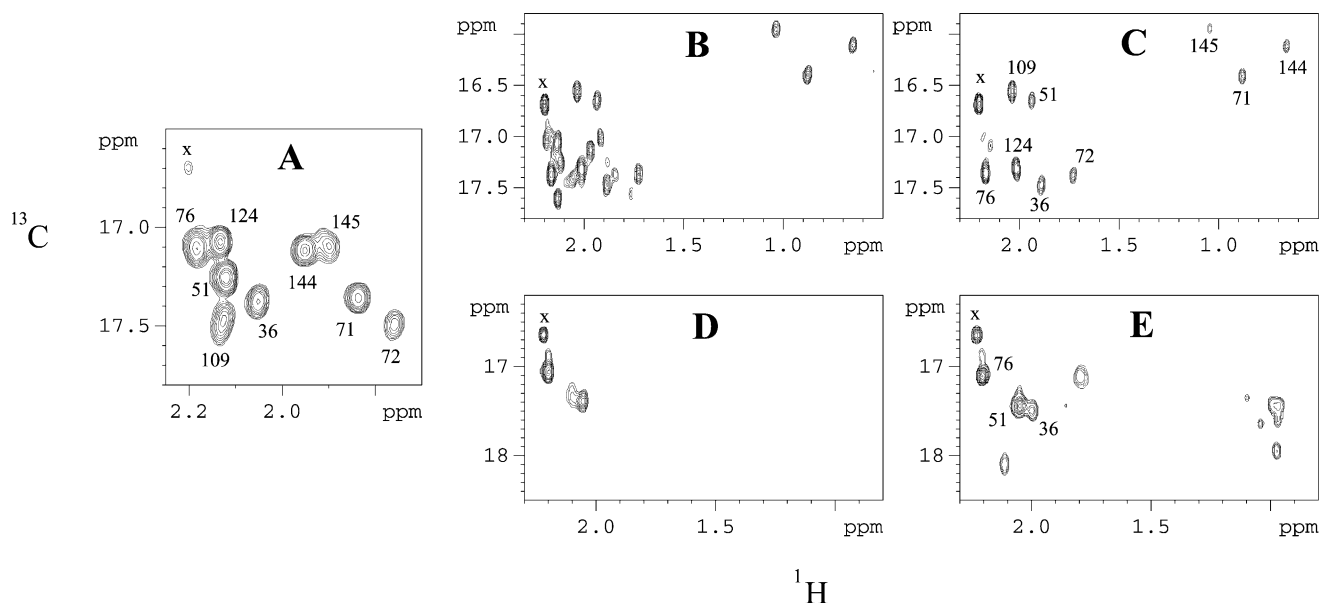


Fig. 3. 2D version of the methionine-edited $^1\text{H}\epsilon\text{-}^{13}\text{C}\epsilon$ spectrum [23] recorded on $^{15}\text{N}/^{13}\text{C}$ -labeled CaM. A: Free CaM. B,C: After addition of 0.5 and 1.0 eq of bOCNCp. D,E: After addition of 1.0 and 2.0 eq of bRCNCp. The peak marked with x probably corresponds to a second conformation of M76 [23]. Assignments of free CaM ϵ -methyl methionine signals were taken from Siivari et al. [33].

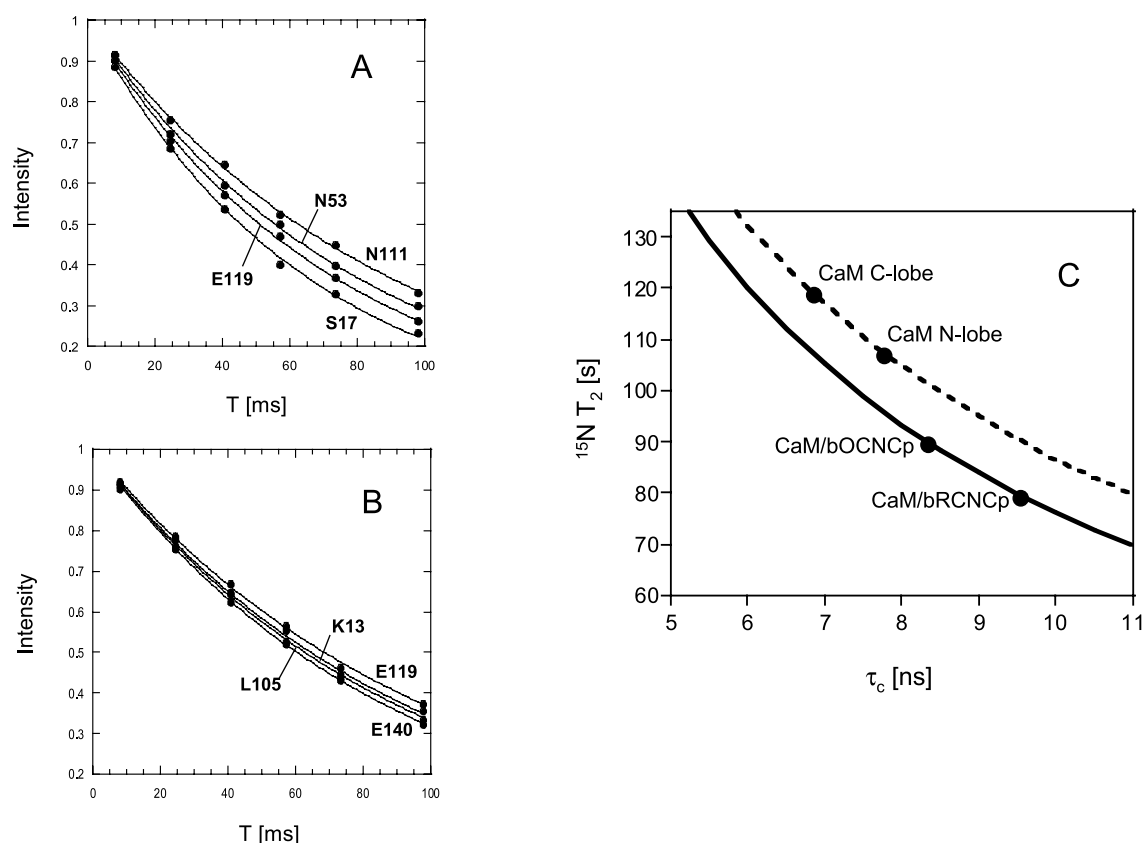


Fig. 4. T_2 fitting of peak intensities vs. delay time for selected residues in CaM-bRCNCp (A) and CaM-bOCNCp (B) complexes. C: Theoretical dependence of $^{15}\text{N } T_2$ for residues showing $S^2=0.85$ at 500 (dashed line) and 700 MHz (solid line).

mode of binding [15–19]. For residues having a low local mobility such as the ones belonging to an α -helix, the T_2 values for the ^{15}N nuclei of backbone amides can be correlated to τ_c using known equations [20]. Fig. 4C shows the theoretical dependence on τ_c of $^{15}\text{N } T_2$ considering $S^2=0.85$ at 500 and 700 MHz. Values for $^{15}\text{N } T_2$ of free CaM at 500 MHz have already been reported [20], and using the curve of Fig. 4C two distinct τ_c of 7.8 and 6.8 ns can be estimated for the N- and C-lobe, respectively. The good agreement with the correlation times calculated by fitting the whole T_2 , T_1 and $^1\text{H}, ^{15}\text{N}$ steady-state NOE dataset (7.12 ± 0.42 and 6.30 ± 0.48 ns) [20] shows that at least for CaM complexes, the analysis here proposed is a suitable tool to estimate rapidly the global τ_c . The observed shorter τ_c for the C-lobe in free CaM is a consequence of its smaller size and of the fact that the two lobes move independently in solution because of the central linker flexibility [20]. The two complexes studied in the present

work show a uniform T_2 for ^{15}N amides in the two lobes, at variance with what is observed in free CaM. This observation suggests that both complexes are compact and that the two lobes are tumbling together, indicating that they are held together through the interaction with the peptides. Moreover, the τ_c for CaM-bRCNCp is around 1.2 ns longer than that estimated for CaM-bOCNCp (Table 1). This difference should roughly correspond to a 2.5 kDa increase in molecular weight for a globular protein in the present conditions of temperature and solvent viscosity [24], thus confirming the binding of two peptides in CaM-bRCNCp as compared to one in CaM-bOCNCp.

4. Discussion

The two peptides interacting with CaM studied in the present work are significantly different in their sequence iden-

Table 1

$^{15}\text{N } T_2$ relaxation times at 35°C for helical residues of the two CaM complexes, reported values for free CaM, and estimated correlation times

	$^{15}\text{N } T_2$ (ms)			τ_c (ns) ^d		
	N-lobe ^a	C-lobe ^b	Overall ^c	N-lobe	C-lobe	Overall
Free ^e	106.5 ± 8.7	119.3 ± 11.1	–	7.8	6.8	–
bOCNCp ^f	88.9 ± 4.0	89.0 ± 4.1	89.0 ± 4.0	8.5	8.5	8.5
bRCNCp ^f	79.0 ± 7.2	78.4 ± 6.7	78.7 ± 7.0	9.6	9.7	9.7

^aCorrelation times estimated using the theoretical curves of Fig. 4.

^bOnly residues 7–17, 29–38, 45–54, 65–74 were considered.

^cOnly residues 82–91, 102–111, 119–128, 138–145 were considered.

^dAll residues used for N- and C-lobe calculation were considered.

^eReported T_2 at 500 MHz [20].

^fMeasured at 700 MHz in this work.

tity. bOCNCp can be regarded as a traditional 1-8-14 calcium-dependent calmodulin binding motif [25]. It has the right sequence to form an amphipathic helix with a series of hydrophobic and positively charged residues exhibiting a complementary topology for the interaction with CaM [22]. Our results confirm this propensity since the complex formed by CaM and bOCNCp presents a 1:1 stoichiometry, a slow kinetics of ligand release, and a compact molecular shape as suggested by the uniform T_2 relaxation times of backbone amide ^{15}N nuclei in the two lobes' helices.

bRCNCp cannot be classified into any of the known CaM binding families, although it shares some common features with the consensus sequence for IQ motifs (BQxxBRGxx-BRxxB, where B stands for an hydrophobic residue) [26]. Although complete IQ motifs do not require calcium to bind CaM, bRCNCp binds CaM in a calcium-dependent manner [6,7]. This observation seems to support the hypothesis that an IQ motif lacking the second part (GxxxR) would bind in a calcium-dependent manner [27]. The sequence of bRCNCp, when analyzed in light of the recently described determinants for IQ motifs binding to calmodulin [28], indicates that bRCNCp shows all the determinants for the binding to one lobe (LQELVK) but lacks most of the key residues for the interaction with the second lobe. Our results indicate that both lobes of CaM are implicated in the interaction with 2 equivalents of bRCNCp. It is only the combination of two molecules of bRCNCp that can provide all the residues needed for an interaction that involves both lobes of CaM.

The same stoichiometry has been found for the binding of a peptide fragment of the petunia glutamate decarboxylase (PGDp) to CaM [29]. The interaction of the two molecules of PGDp occurs simultaneously as indicated by titration [29] and dynamic light scattering studies [30] to form a compact complex. Also in our case the two peptide equivalents of bRCNCp interact with CaM simultaneously leading to the formation of a globular complex. Strikingly, both bRCNCp and PGDp present five negative charges and both lobes of CaM are involved in the two cases. The existence of these two binding motifs confirms the extreme plasticity of CaM in the target recognition process.

The different stoichiometry and binding mode between CaM and bOCNCp and bRCNCp suggest a distinct action mode through which CaM modulates the olfactory and the rod channels. However, the use of peptide fragments to represent the effect of the integral N-terminal domains in their interaction with CaM can be partly questioned. In fact, although the affinity of bOCNCp is almost equal to the EC_{50} , so likely fully describing the CaM modulation of channel activity, that of bRCNCp is 10-fold lower [6–8]. This implies that the binding features and stoichiometry for the interaction of CaM with the entire N-terminus and with the bRCNCp segment are not necessarily coincident. A previous study based on surface plasmon resonance suggested that the high affinity target for CaM in the native rod channel is obtained through the interaction of two N-terminal domains [6]. The present work indicates that the sequence of bRCNCp is able to promote the formation of this hypothetical 2:1 complex. On the other hand, recent studies revealed that the rod CNG channel is formed by three α -subunits and only one β -subunit [31], posing a question on how the 2:1 interaction can be reached. It is possible that a yet unknown fragment of the N-terminus of the channel interacts with CaM together

with the bRCNCp region to form a more stable complex that would account for the observed nanomolar modulation of the channel. Another possibility is that the second binding site observed in the C-terminus of the rod channel [6] plays an active role in the formation of a high affinity target for CaM. Taken altogether, the results presented here unambiguously demonstrate a different mode of action of CaM on the olfactory and rod channels although they point out that it is necessary to enlarge the molecular interaction studies to other regions of the rod channel to elucidate the details of this modulation.

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