Biochemical Journal (2021) **478** 487–491
https://doi.org/10.1042/BCJ20200818

PORTLAND PRESS

Commentary

Simultaneous detection of reciprocal interactions between calmodulin, Ca^{2+} and molecular targets: a focus on the calmodulin-RyR2 complex

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In a recent issue of *Biochemical Journal*, Brohus et al. (*Biochem. J.* **476**, 193–209) investigated the interaction between the ubiquitous intracellular Ca^{2+}-sensor calmodulin (CaM) and peptides that mimic different structural regions of the cardiac ryanodine receptor (RyR2) at different Ca^{2+} concentrations. For the purpose, a novel bidimensional titration assay based on changes in fluorescence anisotropy was designed. The study identified the CaM domains that selectively bind to a specific CaM-binding domain in RyR2 and demonstrated that the interaction occurs essentially under Ca^{2+}-saturating conditions. This study provides an elegant and experimentally accessible framework for detailed molecular investigations of the emerging life-threatening arrhythmia diseases associated with mutations in the genes encoding CaM. Furthermore, by allowing the measurement of the equilibrium dissociation constant in a protein–protein complex as a function of $[Ca^{2+}]$, the methodology presented by Brohus et al. may have broad applicability to the study of Ca^{2+} signalling.

The crucial role of Ca^{2+} as a universal second messenger is largely established and the variety of biochemical pathways directly or indirectly regulated by Ca^{2+} has been extensively investigated. In more recent years, the important contribution of Ca^{2+} signalling in cardiac myocytes has become increasingly clear. Growing evidence has been collected as to its direct involvement in the regulation of action potentials and arrhythmias as well as in the modulation of contractile function, transcription regulation and mitochondrial function [1]. The ubiquitous Ca^{2+} sensor calmodulin (CaM) directly participates in the modulation of the cardiac action potential by binding to and controlling the modulation of the voltage-gated potassium, sodium and calcium channels [2]. CaM is a relatively small (148 amino acids) α-helical protein composed of distinct N- and C-terminal domains separated by a flexible linker region. Each domain contains two Ca^{2+} binding EF-hand motifs (Figure 1) that bind Ca^{2+} with a significantly different affinity. Indeed, in the absence of the target, the C-domain binds the cation much stronger (apparent $K_d \sim 1$ μM) than the N-domain (apparent $K_d \sim 10$ μM) [3]. Positive cooperativity of Ca^{2+} binding occurs within each domain, however, in the absence of the target no cooperativity is detected between the N- and C-domains, which bind Ca^{2+} independently of each other [3]. The high specificity and selectivity of CaM-mediated target activation is the consequence of variations in the number of Ca^{2+} ions required to bind CaM for target activation [4] and of the free energy coupling between Ca^{2+} binding and target binding, which results in changes of cooperativity of Ca^{2+} binding of the CaM-target complex [5,6]. To properly regulate the specific biological process, a tripartite equilibrium between Ca^{2+}, CaM and its target is, therefore, established, which relies on microscopic equilibria that may influence each other in a rather complex fashion.

In cardiomyocytes, the release of Ca^{2+} from the sarcoplasmic reticulum is controlled by the large intracellular Ca^{2+} channel referred to as ryanodine receptor 2 (RyR2) [7]. The complex homotetrameric arrangement of the RyR2 channel has been recently solved by cryogenic electron

Received: 27 November 2020
Revised: 6 January 2021
Accepted: 8 January 2021

Version of Record published:
5 February 2021

Biochemical Journal (2021) 478 487–491
https://doi.org/10.1042/BCJ20200818

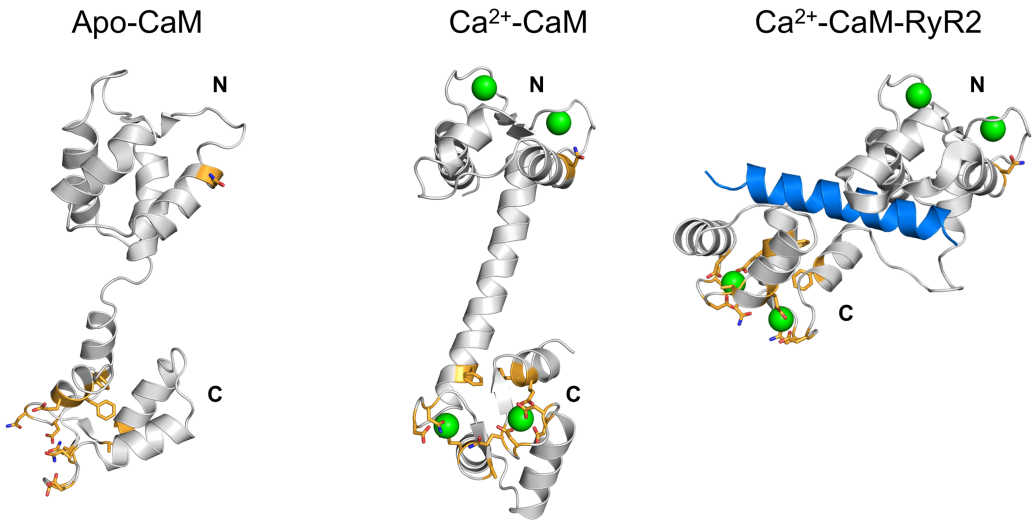

Figure 1. Three-dimensional structure of calmodulin in its apo-form (PDB: 1DMO [25]), Ca^{2+}-bound form (PDB: 1CLL [26]) and in complex with a peptide spanning the 3582–3605 region of RyR2 (PDB: 6Y4O [24]). The N- and C-terminal domains are labelled. Ca^{2+} ions are represented by green spheres, the RyR2 peptide is coloured in blue. Amino acids that have been found to be mutated in genetic cardiac diseases are represented by orange sticks.

microscopy (cryoEM) [8–10], offering an unprecedented insight into the mechanism known as Ca^{2+}-induced Ca^{2+} release (CICR). This phenomenon follows the activation of RyR2 caused by the rise in cytosolic Ca^{2+} upon excitation of cardiomyocytes, and it is necessary to cause myofilament contraction [1]. CaM modulates the activity of RyR2 by inhibiting the release of Ca^{2+} in a Ca^{2+}-dependent manner, a mechanism that is achieved by selective binding to specific regions of the channel with a 1 : 1 stoichiometry, during [Ca^{2+}] oscillations in the ∼0.2 to 200–400 μM range (at diastole and during CICR and systole, respectively) [11,12]. In particular, it has been established that apo-CaM has either no effect or inhibitory effect on RyR2, while Ca^{2+}-CaM inhibits the channel [13].

The full characterization of the mechanisms underlying the Ca^{2+}-dependent binding of CaM to RyR2 is, therefore, crucial to achieve a complete molecular understanding of processes occurring in cardiomyocytes. This is especially apparent in light of the relatively recent discovery that mutations in the genes encoding CaM are associated with ventricular tachycardia and sudden cardiac death [14]. To date, eighteen point mutations in either *CALM1*, *CALM2* or *CALM3*, all genes independently encoding CaM in the human genome, have been associated with catecholaminergic polymorphic ventricular tachycardia (CPVT), long QT syndrome or idiopathic ventricular fibrillation [15,16] (Figure 1). This surprising discovery contrasting with the high level of evolutionary conservation of CaM led to the definition a new branch of pathology named *calmodulinopathy*, with specific clinical phenotypes affecting mostly young individuals [17].

In a recent work published in the *Biochemical Journal*, Brohus et al. [18] unveiled the molecular details of CaM-RyR2 interaction by identifying the CaM-binding domain in RyR2 at varying calcium concentration ([Ca^{2+}]). This is not a trivial task, as the affinity of CaM for Ca^{2+} has been shown to greatly depend on the target, often significantly increasing in its presence [19–21]. Moreover, the binding of CaM to its target is itself governed by a specific equilibrium, which needs to be investigated independently and can also be influenced by the presence of Ca^{2+}. Therefore, one normally needs to perform a long series of titration experiments, in which either the protein concentration is kept fixed and that of Ca^{2+} is varied, or the other way around. Inspired by the quantitative approach developed by Dagher et al. [22], Brohus et al. designed a fluorescence anisotropy (FA)-based titration method that permitted a fast exploration of the Ca^{2+}-dependency of the interaction between CaM and four putative RyR2 binding domains, while varying [Ca^{2+}] over a broad range of values, including the physiological levels in cardiomyocytes.

Peptides mimicking the four putative CaM-binding domains in RyR2 were synthesized with a fluorescent N-terminal 5-carboxytetramethylrhodamine (5-TAMRA) label and used in titration experiments with CaM, or its individual N- and C-domains, at 16 discrete values of [Ca^{2+}] [18]. The assay detects the change in the FA

Biochemical Journal (2021) **478** 487–491
https://doi.org/10.1042/BCJ20200818

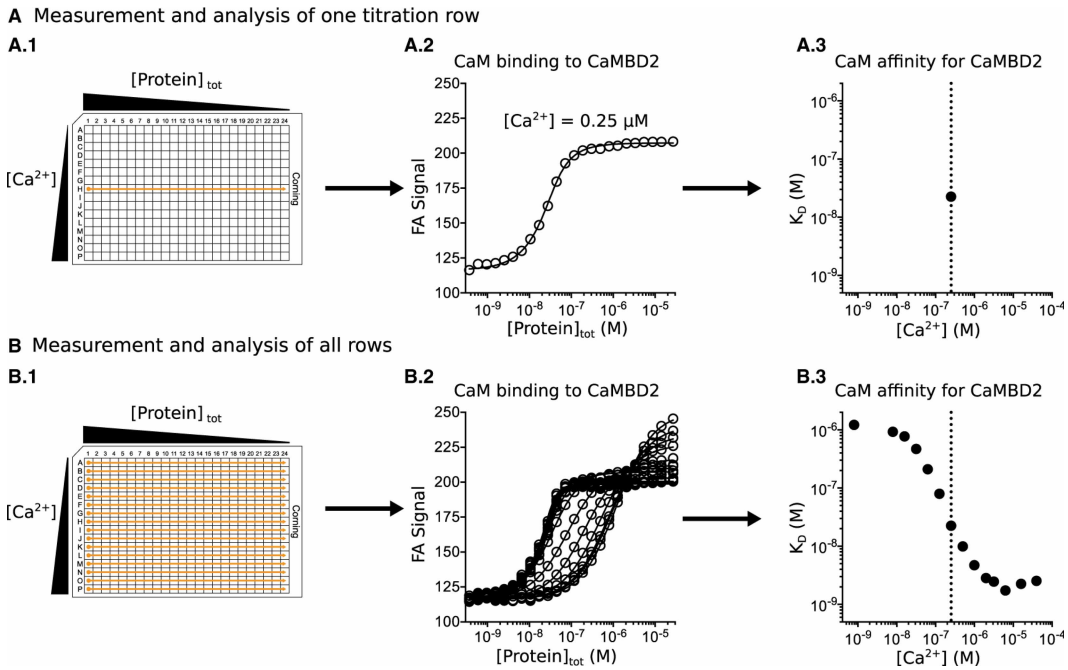

Figure 2. Principle of the bidimensional titration assay based on FA measurements developed by Brohus et al. [18].
(**A**) Simplified measurement and analysis example. Each row in the microtiter plate (**A.1**) contains a titration series of RyR2 peptide with decreasing protein concentration (columns 1–24) at one fixed [Ca^{2+}]. The FA signal from each well in the row as a function of the total protein concentration generates the titration curve shown in (**A.2**), by which the relative K_D for the protein binding to peptide at that particular [Ca^{2+}] is obtained by fitting (**A.3**). (**B**) The fitting procedure is repeated for the titration curves from each row (**B.1–2**), thus giving the K_D as a function of [Ca^{2+}] (**B.3**). CaMBD2 represents the peptide spanning the 3581–3607 region of RyrR2. Adapted from ref. [18].

signal of the 5-TAMRA-labelled peptide occurring upon CaM (or CaM domain) binding. Since a 16 × 24 wells plate is used (Figure 2), a thorough range of concentration of CaM can be tested (23 serial dilution steps across the columns of one plate row) while keeping the concentration of the interacting peptide constant (∼50 nM) and varying the [Ca^{2+}] discretely in the 16 rows, in the 0.3 nM–400 µM range. Overall, each microtiter plate allows for 24 titration points for CaM concentration at each of the 16 different values of [Ca^{2+}], thus providing 16 CaM-peptide titration curves (Figure 2) in less than 50 min. For most peptides used to mimic the four CaM-binding domains in RyR2 the binding data fit well a simple 1 : 1 interaction model, and the plot of the FA signal as a function of protein concentration at each [Ca^{2+}] (row) showed a sigmoidal trend representing the fractional saturation of the peptide with CaM, finally yielding an apparent K_D value at a specific [Ca^{2+}]. By fitting each row-wise titration curve, K_D as a function of [Ca^{2+}] could be obtained by nonlinear regression (Figure 2). The authors could also estimate the apparent affinity for Ca^{2+} of the CaM-peptide complexes by monitoring FA signals as a function of [Ca^{2+}] (column-wise curves) at specific CaM : peptide ratios, ensuring an excess of CaM. By performing a curve fitting to an empirical Hill model, they could estimate the apparent K_D of Ca^{2+}-binding to the CaM-peptide complex and the cooperativity of the process described by the Hill coefficient [23].

The well-designed experiments reported by Brohus et al. [18] permit a wide set of different conditions to be monitored simultaneously, and provide a direct methodology to test which domain of CaM preferentially binds to a specific RyR2 region over a physiological range of [Ca^{2+}]. Based on their exhaustive screening, the authors were able to build up a detailed dynamic model of domain-wise interaction of CaM with RyR2, and proposed a specific binding scheme that follows [Ca^{2+}] increases from 3 nM up to >10 µM. They concluded that the C-domain of CaM is constitutively bound to the 3581–3611 region of RyR2 in a Ca^{2+}-saturated state, while the N-terminal domain may serve as a dynamic Ca^{2+} sensor that could either bridge non-adjacent regions of RyR2 or clamp down onto the same interacting region as the C-domain. In a subsequent recent work [24], the same

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authors in collaboration with other groups solved the X-ray and NMR structures of CaM in complex with a peptide covering the same 3581–3611 RyR2 region. The complex showed very similar structural properties when comparing wild-type CaM and the N53I mutant associated with CPVT, which demonstrates a tight binding of the peptide to both variants that involves both the N and C-terminals; moreover, the study suggested an alteration of the interaction of the N-terminal domain of CaM with RyR2 induced by the mutant [24]. Recent cryoEM determinations are substantially in line with these findings [8]. Apo-CaM and Ca^{2+}-CaM bind to distinct but overlapping sites in an elongated cleft formed by the handle, helical and central domains of RyR2 [8]. These high-resolution structures confirmed that the shift in CaM-binding sites on RyR2 is controlled by Ca^{2+} binding to CaM rather than to RyR2, which substantially matches the mechanism proposed by Brohus et al. [18].

The fine regulation of the heartbeat depends critically on very precise affinity and kinetics of the RyR2-CaM-Ca^{2+} reciprocal interactions. The work reported by Brohus et al. constitutes a significant methodological and conceptual advance in the virtually simultaneous investigation of this tripartite equilibrium. Besides shedding light on the physiological processes in cardiomyocytes, the study has a more general relevance as, with some modifications, it allows the simultaneous detection of the equilibrium dissociation constant in a protein–protein complex as a function of intercellular [Ca^{2+}]. This has a great potential for the study of Ca^{2+} signalling in a variety of biochemical pathways, especially considering that CaM is known to interact with more than 300 target proteins.

Competing Interests

The author declares that there are no competing interests associated with this manuscript.

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Open access for this article was enabled by the participation of University of Verona in an all-inclusive *Read & Publish* pilot with Portland Press and the Biochemical Society.

Acknowledgements

This work was supported by a grant from the Italian Ministry for Education, University and Research (PRIN 2017 201744NR8S 'Integrative tools for defining the molecular basis of the diseases'). I would like to thank Dr. Giuditta Dal Cortivo for critically reading the manuscript.

Abbreviations

CICR, Ca^{2+}-induced Ca^{2+} release; CaM, calmodulin; CPVT, catecholaminergic polymorphic ventricular tachycardia; cryoEM, cryogenic electron microscopy; FA, fluorescence anisotropy; RyR2, cardiac ryanodine receptor; TAMRA, 5-carboxytetramethylrhodamine.

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