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A general framework to characterize inhibitors of calmodulin: Use of calmodulin inhibitors to study the interaction between calmodulin and its calmodulin binding domains ☆

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ABSTRACT

The prominent role of Ca^{2+} in cell physiology is mediated by a whole set of proteins involved in Ca^{2+}-signal generation, deciphering and arrest. Among these intracellular proteins, calmodulin (CaM) known as a prototypical calcium sensor, serves as a ubiquitous carrier of the intracellular calcium signal in all eukaryotic cell types. CaM is assumed to be involved in many diseases including Parkinson, Alzheimer, and rheumatoid arthritis. Defects in some of many reaction partners of CaM might be responsible for disease symptoms. Several classes of drugs bind to CaM with unwanted side effects rather than specific therapeutic use. Thus, it may be more promising to concentrate at searching for pharmacological interferences with the CaM target proteins, in order to find tools for dissecting and investigating CaM-regulatory and modulatory functions in cells.

In the present study, we have established a screening assay based on fluorescence polarization (FP) to identify a diverse set of small molecules that disrupt the regulatory function of CaM. The FP-based CaM assay consists in the competition of two fluorescent probes and a library of chemical compounds for binding to CaM.

Screening of about 5300 compounds (Strasbourg Academic Library) by displacement of the probe yielded 39 compounds in a first step, from which 6 were selected. Those 6 compounds were characterized by means of calorimetry studies and by competitive displacement of two fluorescent probes interacting with CaM. Moreover, those small molecules were tested for their capability to displace 8 different CaM binding domains from CaM. Our results show that these CaM/small molecules interactions are not functionally equivalent.

The strategy that has been set up for CaM is a general model for the development and validation of other CaM interactors, to decipher their mode of action, or rationally design more specific CaM antagonists. Moreover, this strategy may be used for other protein binding assays intended to screen for molecules with preferred binding activity. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

1. Introduction

Ca^{2+} acts as a global second messenger in cell physiology and its role is mediated by a whole set of proteins involved in Ca^{2+}-signal generation, deciphering and arrest. Among these intracellular proteins, calmodulin (CaM), a fundamental calcium-modulated protein, has been selected along evolution as a calcium hub in eukaryotes. It exerts a major role in regulating both calcium homeostasis and intracellular signaling. CaM is assumed to be involved in many diseases including Parkinson, Alzheimer, and rheumatoid arthritis [1–3]. CaM interacting small molecules have been sought and largely used in biological laboratories, mainly in the 80s. However, a substantial number of them tend to do so with low specificity and uncertain stoichiometry [4]. Several classes of hydrophobic small molecules including phenothiazines, naphthalene sulphonamides, imidazoles and dihydropyridines bind to CaM with affinities in the micromolar range and inhibit its ability to activate target enzymes. Thus, to find tools allowing dissection and investigation of CaM-dependent pathways, it may be more promising to concentrate the search on pharmacological interference between CaM and its target proteins [5]. The co-crystallization of CaM with various small antagonists illustrates the plasticity of CaM, with its ability to adapt its shape to different molecules [6–8].

Recently, the use of CaM antagonists resumed due to the renewal of chemical biology. Moreover, CaM could be an interesting therapeutic target in some types of cancers and inflammatory diseases [9–11].

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Evidence has been obtained showing that specific antagonists of the Ca2+/CaM complex inhibit the growth of a variety of tumor cells [9,12,13].

In a previous study, a fluorescence polarization (FP) based screen of a chemical library allowed us to disclose a set of diverse compounds that can be used as tools to decipher the role of CaM in regulating intracellular calcium signaling [14]. In this paper, we first describe the application of this competitive FP-based assay that uses the fluorescent probes 16B05 (probe S1) or 17F07 (probe S3) characterized previously [14] to the screening of CaM with the collection of chemical compounds of the Strasbourg Academic Library. The CaM binding characteristics of a subset of the compounds disclosed were further studied using biophysical approaches. Finally, we analyze the ability of such compounds to inhibit the interaction between CaM and several CaM-binding domains using an assay described previously [15]. Comparing the behavior of the CaM antagonists reveals that the small molecules are not functionally equivalent. These findings indicate that analyzing data obtained by using CaM antagonists to evaluate the role of CaM in a cellular event must be done with caution. On the other hand, those results rejuvenate the pharmacology of CaM.

2. Experimental section

2.1. Materials

All chemicals were obtained from commercial suppliers and used without further purification. DMSO, KCl, and Hepes were purchased from Sigma. $CaCl_2$ was from Fluka. Ultra-pure water (Milli Q instrument from Millipore Corp., MA, USA) was used for the aqueous solutions. The assays were carried out with synthetic CaM (SynCaM), a hybrid between mammalian and plant CaM [16] or with human CaM. Fluorescent probes CHPO 199-5-B05 and CHPO 199-6-F07 ortho isomers (16B05 ortho and 17F07 ortho, respectively) were selected from the fluorescent polarization screening assay applied to CaM and characterized biophysically [14]. Peptides were synthesized by Jena as previously described [15]. Their purity is higher than 85%. 10 mM stock solutions of peptides in DMSO were prepared.

2.2. Strasbourg Academic Library

The screened chemical library belongs to the Platform of Integrative Chemical Biology of Strasbourg (PCBIS) and is a member of the core facilities of the CNRS called "National Chemical Library" (http://chimiotheque-nationale.enscm.fr/) which collects and itemizes all molecules available from the participating laboratories in France. The chemical library includes more than 5300 molecules itemized and classified in the database (MDL® ISIS/Base and Activity Base) and conditioned in master microtube plates applying industrial standards. They are stored in 96 well microplates under normalized conditions, at a concentration of about 10 mM in DMSO for automated screening assays. The selected chemical compounds are royalty-free and their methods of synthesis are known. The purity of the components is more than 80% (characterized by LC–MS). Addition of natural products and extracts increases the diversity of the library. The Strasbourg Academic Library is the initiator of the French National Chemical Library. Since January 16th 2007, the chemical library platform has obtained the certificate of approval ISO 9001:2000.

The molecules identified in this study and their physical and chemical characteristics are available upon request from Pascal Villa and Bruno Didier for non-commercial purpose. All the molecules have been characterized by LC–MS and their purity is greater than 95%.

2.3. Spectroscopic measurements

Steady-state absorption spectra were recorded on a NanoDrop ND-1000 apparatus (Labtech) spectrophotometer in order to determine the protein concentrations. Extinction coefficients of 1650 M^{-1} cm^{-1} were used for SynCaM (VU1) [15]. All spectra were corrected for lamp intensity variations and background. All the measurements were carried out at 20 °C.

2.4. CaM screening assay based on fluorescence polarization (FP)

Competitive FP-screening assays were performed with each of the two probes (16B05 ortho isomer and 17F07 ortho isomer) using a Victor3 apparatus (Perkin-Elmer Life and Analytical Sciences, Boston, MA), handled by a Biomek 2000 robot, conducted in Corning Costar 96-well black polystyrene flat-bottomed plates (Model 3686; Corning, Acton, MA) and carried out at 25 °C. The Strasbourg Academic Library was screened by adding to each well, successively, 15 μL of a library compound at 20 μM in the assay buffer (50 mM Hepes, 150 mM KCl, 10 μM $CaCl_2$ pH 7.5) and 15 μL of the mixture composed of 4 μM of CaM and 0.2 μM of the fluorescent probe prepared in the same buffer. Control wells were planned for each plate, by means of 6 wells containing the fluorescent probe (0.1 μM) bound to CaM (2 μM) in a final volume of 30 μL and 6 wells containing 0.1 μM of probe alone (unbound probe) in 30 μL of the solution buffer. The plate was stirred and incubated for 5 min at room temperature. The equilibrium between a fixed concentration of the fluorescent probe and its increasing free concentration, as a result of the probe displacement by library compounds, was monitored by fluorescence polarization (FP). The polarization degrees were measured with an excitation wavelength set at 530 nm (bandwidth 7 nm) and an emission wavelength set at 610 nm (bandwidth 10 nm). The fluorescent probes used (16B05 (probe S1) or 17F07 (probe S3)) consist of two pharmacophores fused to the fluorescent molecule lissamine [14].

2.5. Data analysis

The polarization degree is defined by the equation $P=(I_{//}-I_{\perp})/(I_{//}+I_{\perp})$ where P is the fluorescence polarization degree, $I_{//}$ and $I_{\perp}$ are the fluorescence intensities of the light emitted, respectively, parallel (//) and perpendicular ($_{\perp}$) with respect to the plane of linearly polarized excitation light. For each plate, background correction was done by subtracting blank parallel and perpendicular components (the means of 2 wells containing only buffer) from the respective intensities. When calculation are performed on FP data, anisotropy rather then polarization degree values are used, because they can be combined additively [17]. Anisotropy values (A) were obtained from polarization (P) as follows [17]:

$$A = \frac{2P}{3-P}.$$

2.6. Calculation of the Z′-factor

To assess the suitability of the assay for high-throughput screening applications, the Z′ value was calculated using the equation: $Z' = 1 - (3SD_{bound} + 3SD_{free})/(mP_{bound} - mP_{free})$, where SD is the standard deviation and mP is the fluorescence polarization expressed as $P \times 1000$, where P stands for the polarization degree. The "bound state" is represented by the fluorescent probe bound to the protein and the "free state" is represented by the unbound fluorescent probe prepared in the solution buffer.

2.7. Biophysical characterization of CaM selected interactors

FP-titrations were performed using a Victor3 apparatus (Perkin-Elmer Life and Analytical Sciences, Boston, MA) and carried out at 25 °C as most of the previous biophysical studies on calmodulin have been done at this temperature. Binding assays were conducted