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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

in Corning Costar 96-well black polystyrene flat-bottomed plates (Model 3686; Corning, Acton, MA) and handled by a Biomek 2000 robot. The FP-titrations were planned by adding to each well, successively, 15 μL of a mixture of SynCaM (2 μM) and the 16B05 ortho isomer probe (0.2 μM) in the assay buffer (50 mM Hepes, 150 mM KCl, $CaCl_2$ 10 μM pH 7.5) and 15 μL of CaM interactors, in the same buffer with final concentrations ranging from 0.5 to 500 μM in a final volume of 30 μL. In experiments performed with 17F07 (probe S3), CaM concentration were in the 5 μM range. The final DMSO concentration in the assay buffer was less than 1%. The plate was shaken and incubated for 5 min at room temperature. The polarization degrees (P) were measured with an excitation wavelength set at 530 nm (bandwidth 7 nm) and an emission wavelength set at 610 nm (bandwidth 10 nm). For each plate, P were measured versus control wells containing 16B05 or 17F07 ortho isomer bound to SynCaM and the ortho isomer probe alone (unbound probe). Probe displacement by different concentrations of CaM interactors was monitored by steady-state fluorescence polarization. Equilibrium parameters were determined by non-linear curve fitting using the general framework described by Roehrl et al. [17].

2.8. Isothermal microcalorimetry titration of CaM with selected interactors

The thermodynamic parameters of CaM inhibitors binding to SynCaM were measured using a ITC200 instrument (MicroCal, Northampton, MA, USA) as described previously [18]. Experiments were carried out at 25 °C in 50 mM Hepes buffer in the presence of 2 mM $CaCl_2$ and 2% DMSO, pH 7.5. 2-μL aliquots of CaM inhibitors were injected into a 0.2 mL cell containing the SynCaM solution to achieve a complete binding isotherm. Concentration of SynCaM in the cell was 15 μM and concentration of CaM inhibitors in the syringe ranged from 0.15 to 0.75 mM. The heat of dilution was measured by injecting the ligand into the buffer solution or by additional injections of ligand after saturation; the values obtained were subtracted from the heat of reaction to obtain the effective heat of binding. ITC baseline was always stable without any signs of precipitation. The resulting titration curves were fitted by MicroCal Origin software using the model of two sets of independent binding sites. Among the different models provided by MicroCal origin software, the model of two sets of independent sites gave the best fit. Affinity constants (K_a), binding stoichiometry (N) and enthalpy (ΔH) were determined by a non-linear regression fitting procedure. ITC measurements for each CaM inhibitor have been repeated at least three times and yielded similar thermodynamic parameters.

2.9. FP-competitive binding assay between CaM-interactors and synthetic peptides

The interaction between CaM and TAMRA-tagged fluorescent peptides in the presence of interactors was studied by FP-measurements using a Victor3 (Perkin-Elmer Life and Analytical Sciences, Boston, MA) at 25 °C. For this purpose, peptides at 0.1 μM were titrated with 1 μM of CaM and various concentrations of interactors (1–2–4–8–16–32–64–125–250–500–1000 μM). Assays were conducted in a 30 mM MOPS buffer, 100 mM KCl and 100 μM CaCl2, pH 7.2.

Solutions composed of 0.2 μM of peptides and 2 μM of CaM were distributed robotically (BiomeK 2000, Beckman Coulter) in 96 well microplates (Corning Costar 3686, Acton, MA). Stock solutions of interactors (50 mM in DMSO) were prepared and were serially diluted to obtain concentrations ranging from 0.02 to 2000 μM. The binding assays were performed by adding robotically (BiomeK FX, Beckman Coulter) 7.5 μL of interactor to each well containing 7.5 μL of fluorescent peptide and CaM mix. The final interactor concentration thus ranged from 1 μM to 1000 μM for a peptide concentration of 0.1 μM and a CaM concentration of 1 μM. The plate was mixed and incubated for 5 min at room temperature. The polarization degrees (P) were measured with an excitation wavelength set at 544 nm (bandwidth 15 nm) and an emission wavelength set at 600 nm (bandwidth 10 nm). For each plate, FP-measurements were performed with control wells containing the unbound fluorescent peptide. During titration, the equilibrium ratio of free over total fluorescent probe concentration was monitored by fluorescence polarization. The half maximal effective concentration (IC50) of interactors able to inhibit peptide binding to CaM was calculated by fitting the sigmoid dose-dependent with the Hill equation (the Hill equation is a phenomenological equation that was fitting reasonably well our experimental data points):

$$P = \frac{P_{max} + P_{min} * \left(\left(\frac{1}{IC50} * x\right)^{n_h}\right)}{1 + \left(\left(\frac{1}{IC50} * x\right)^{n_h}\right)}$$

where x is the concentration of CaM interactor, IC50 stands for the concentration of interactor that gives 50% binding inhibition and n_h is the Hill coefficient that describes the slope of the curve when the concentration of antagonist is equal to the IC50. P_{max} is the maximal polarization under the experimental conditions used. As the concentration of CaM is 1 μM and the concentration of labeled peptide is 0.1 μM, the P_{max} value is an indication of the affinity between CaM and a given peptide. P_{max} may vary from one experiment to the other due to small changes in CaM concentration. P_{min} represents the minimum polarization reached in the experiment. In the present experiments, P_{min} is equal to the polarization of the free tagged peptide, indicating that the different CaM interactors are able to dissociate the complexes formed between CaM and its binding domains.

3. Results and discussion

3.1. Identification of CaM interactors by screening the Strasbourg Academic Library and selection of compounds for further use in CaM/CaM peptide interaction studies

A previous screen of the Prestwick chemical library using fluorescence polarization disclosed 11 chemical molecules interacting with CaM, among which is trifluoperazine, one of the well known CaM antagonists [14]. Using the same approach, the Strasbourg Academic Library consisting of 5300 compounds, was screened at 10 μM final concentration with 0.1 μM fluorescent probe (16B05 (probe S1) or 17F07 (probe S3)) bound to 2 μM SynCaM in the presence of 10 μM Ca^{2+} in a final volume of 30 μL per well. The experimental setup is based on competitive fluorescence polarization that monitors the change in fluorescence polarization of a given probe (S1 or S3) upon binding of a compound of the chemical library to SynCaM. The free fluorescent probes show low polarization values, whereas the bound probes exhibit high values.

The performance of the assay was measured by analyzing statistical quality parameters, such as the Z′ factor [19]. The Z′ factor quantifies the difference that separates the signals corresponding to the polarization degree of the bound ligand and the free ligand in the FP-screening assay and accounts for the observed variability. A Z′ value of 0.85 was calculated from our data. Typically, an assay in which $0.5 \leq Z' \leq 1$ is considered excellent.

Screening the Strasbourg Academic Library revealed 39 molecules able to interact with CaM. Among those 39 positive hits, six were selected as they were distributed into two homogenous chemical families, each composed of three molecules. Such molecules are adequate tools for deeper analysis of the CaM interaction properties. Additionally, trifluoroperazine (TFP), an extensively studied molecule that has been largely employed as CaM inhibitor, was used as control (Fig. 1).

The three N-(2-naphthylmethyl) ethane-1, 2-diamine derivatives (Fig. 1 upper panel) compose our first family of molecules. The members differ by the presence on the benzene ring of either an −OH or a −Cl group.

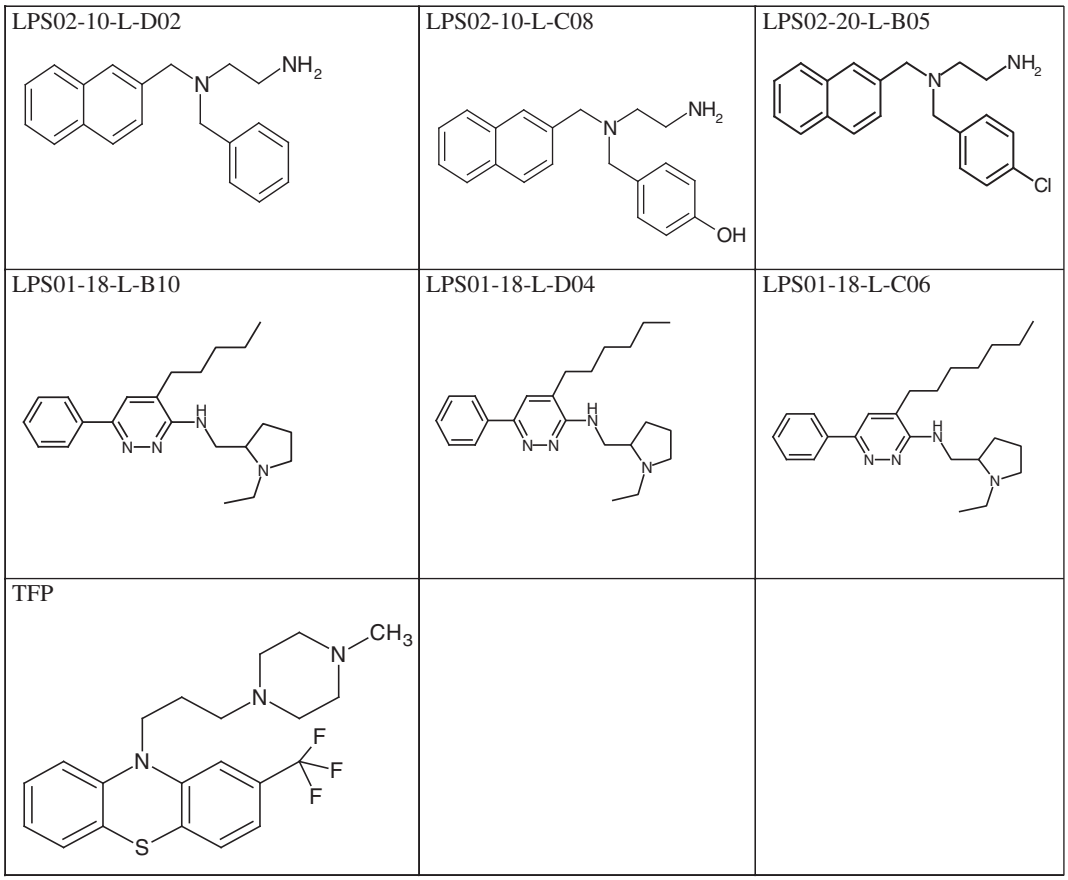

Fig. 1. Chemical structures of the CaM interactors selected after screening the Strasbourg Academic Library and hit validation. The upper panel presents the structures of the molecules forming family 1, the middle panel presents those forming family 2 molecules and the lower panel corresponds to the structure of trifluoroperazine.

The three amino alkyl pyridazine derivatives (Fig. 1, middle panel) constitute family 2. They differ by the length of the alkyl chain with 5, 6 or 7 carbons.

3.2. Interaction characteristics of the CaM interactors evaluated using fluorescence polarization titration experiments

To characterize the mode of binding of the CaM antagonists, we used the fluorescent polarization assay previously described [14]. The two fluorescent probes S1 and S3 that bind to CaM with high affinity in a calcium dependent manner were competed with the aforementioned selected CaM antagonists (Fig. 1). The level of probe binding to CaM was monitored by fluorescence polarization. In our experimental conditions, the S1 and S3 probes bind to one site on CaM with dissociation constants of 0.16 μM and 0.89 μM, respectively. Under the experimental conditions used, CaM and probe concentrations were kept constant (from 0.5 to 2 μM in the different experiments for CaM and 0.1 μM for the probe), and the calmodulin interactor concentrations ranged from 0.5 to 500 μM. Monitoring the fluorescence polarization changes of the probe during titration of the CaM-probe complex with the different CaM interactors gives information on whether the antagonists bind to the same site as the fluorescent probe or to one or several additional different sites (Fig. 2).

As illustrated in Fig. 2, two possibilities were considered for the interaction of CaM with the chemical compounds:

1) A simple competition with the S1 or S3 probes. Such competition is described by the equation presented in Dagher et al. [14]. In this case, as the dissociation constant for each probe is known from previous experiments (namely 0.16 μM for probe S1 and 0.89 μM for probe S3), the dose response curves established from titration data give access to the dissociation constant Ki between CaM and each CaM interactor. We assume that the two probes bind to the same or neighboring domains as the calcium dependency of the probe binding is similar (data not shown).
2) An allosteric mechanism where the antagonist binds to one or several sites that differ from the probe site. Even in this case, the apparent titration by a CaM antagonist appears as a competition with the probe.

As presented in Fig. 3, the displacement of probes S1 and S3 from CaM induced by the different CaM antagonists is reasonably fitted by a competitive model between the probes and the different antagonists. This competitive model was chosen as it allowed fitting the raw data with the minimum number of parameters. However, those results do not exclude the possibility of other binding sites for the antagonists. Indeed, either the molecule competes directly with the probe or the binding site of the molecule is negatively coupled to the probe binding site. The parameters used to fit the experimental data, as well as the residual mean square (RMS) illustrating the quality of the fit, are summarized in Table 1.

In the first group, the molecule with the chloride group on the benzene ring (B05) exhibits an affinity almost 5 fold higher than the one with the benzene (D02) ring and 10 fold higher than the molecule with a phenol (C08). This result is reminiscent of the properties of the couple of CaM antagonists designed by Hidaka [20], namely W7 and W5. In our hand, W7 has an affinity 10 times lower than the molecule B05 (data not shown). In the second group, the molecules with the alkyl chain of length 6 or 7 present almost the same affinity whereas

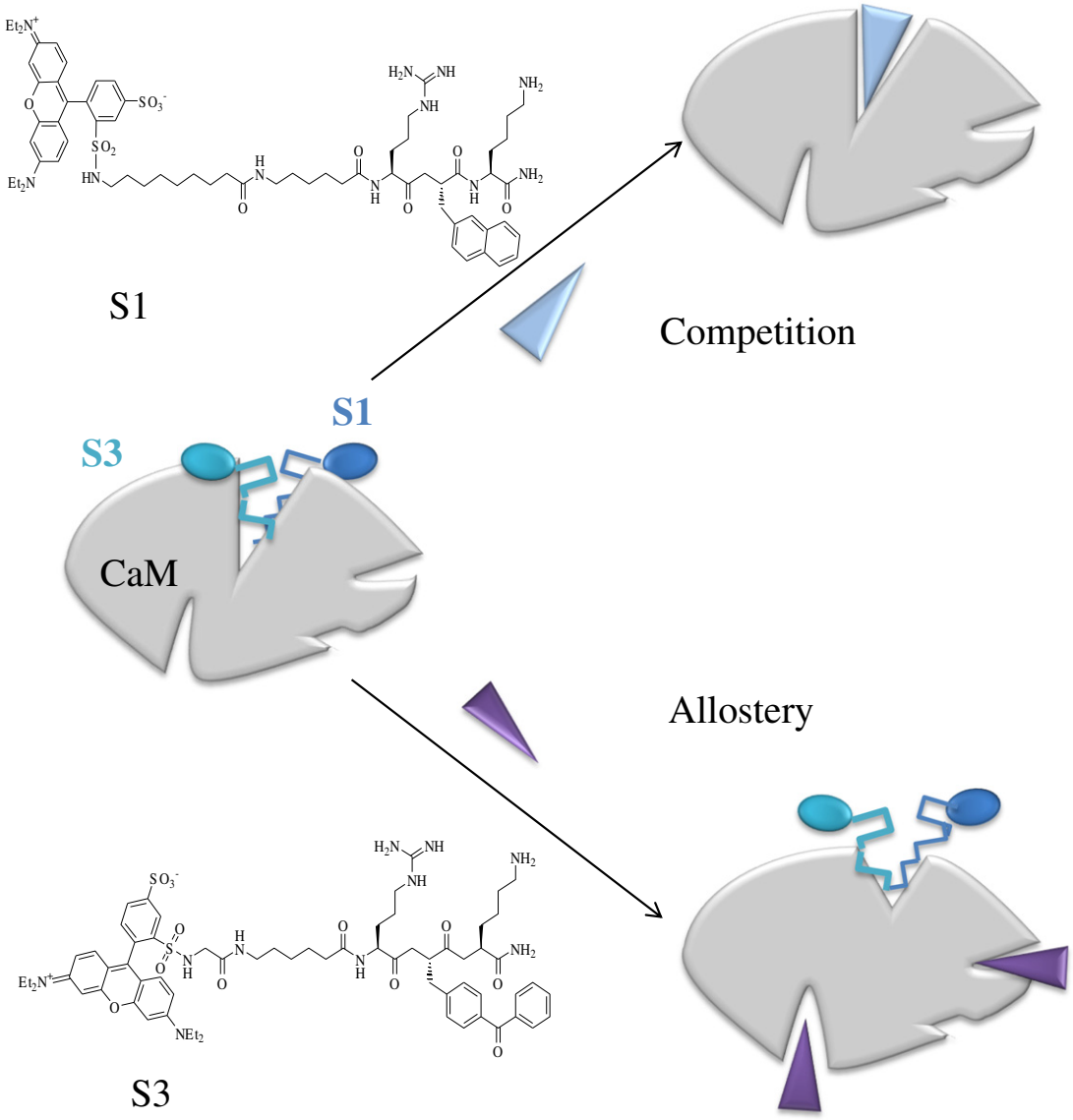

Fig. 2. The two possible schemes considered for the interaction of CaM with its antagonists. The probes S1 or S3 bound to CaM can be displaced by a CaM interactor (arrowhead) either by direct competition (blue arrowhead) or by an allosteric mechanism (purple arrowhead).

the molecule with an alkyl chain of length 5 has an affinity nearly 5 times lower.

By comparison, trifluoroperazine has a Ki of 1 μM (data not shown) in agreement with values reported in the literature.

So far, our study allowed us to characterize two groups of three molecules, each group belonging to a homogeneous chemical class. Those molecules exhibit different affinities for CaM and can thus be used as pharmacological tools allowing to differentiate cellular effects due to CaM or to off target proteins. Indeed, we may expect that when used to inhibit a cellular event involving CaM, for each family, the molecule with the highest affinity would be more potent than the molecule with the lowest affinity. In other terms, for the family 1, the molecule B05 should show more efficacy than the molecule C08. Following the same rationale for the family 2, the molecule C06 must be more potent that the molecule B10.

Moreover, following this simple rationale, if the molecules in the two chemical families, behave similarly, we may expect that B05 (family 1) would be more potent than the molecule C06 (family 2). More generally, we may expect that the potency of each molecule to inhibit a cellular event involving CaM will exhibit the same ranking than the binding constants of that molecule for CaM.

3.3. Thermodynamic approach to characterize the binding mechanism of CaM-interactors

In order to get more insight into the molecular mechanism of binding of the selected molecules to CaM, we monitored their interaction by microcalorimetry in a medium without KCl and containing 2% DMSO. The experimental conditions were designed to get reproducible data with a good signal to noise ratio.

The thermodynamic parameters for each molecule obtained from the titration curves presented in Fig. 4 are given in Table 2.

The calorimetric study qualitatively confirms the results obtained by fluorescence polarization in terms of ranking of the binding affinities of the different molecules for CaM. Under the experimental conditions used, the molecules of family 1 exhibit one high affinity binding site in the nM range and two low affinity sites in the μM range. Similarly, the molecules of family 2 exhibit one high affinity binding site in the 10 nM range and 3 sites in the μM range. The affinity increases as the alkyl chain elongates. In all cases, the binding is entropically and enthalpically driven. For the different molecules, the relative ranking of the affinity constants is similar when considering the binding constants of the high affinity sites obtained by calorimetric titration or the

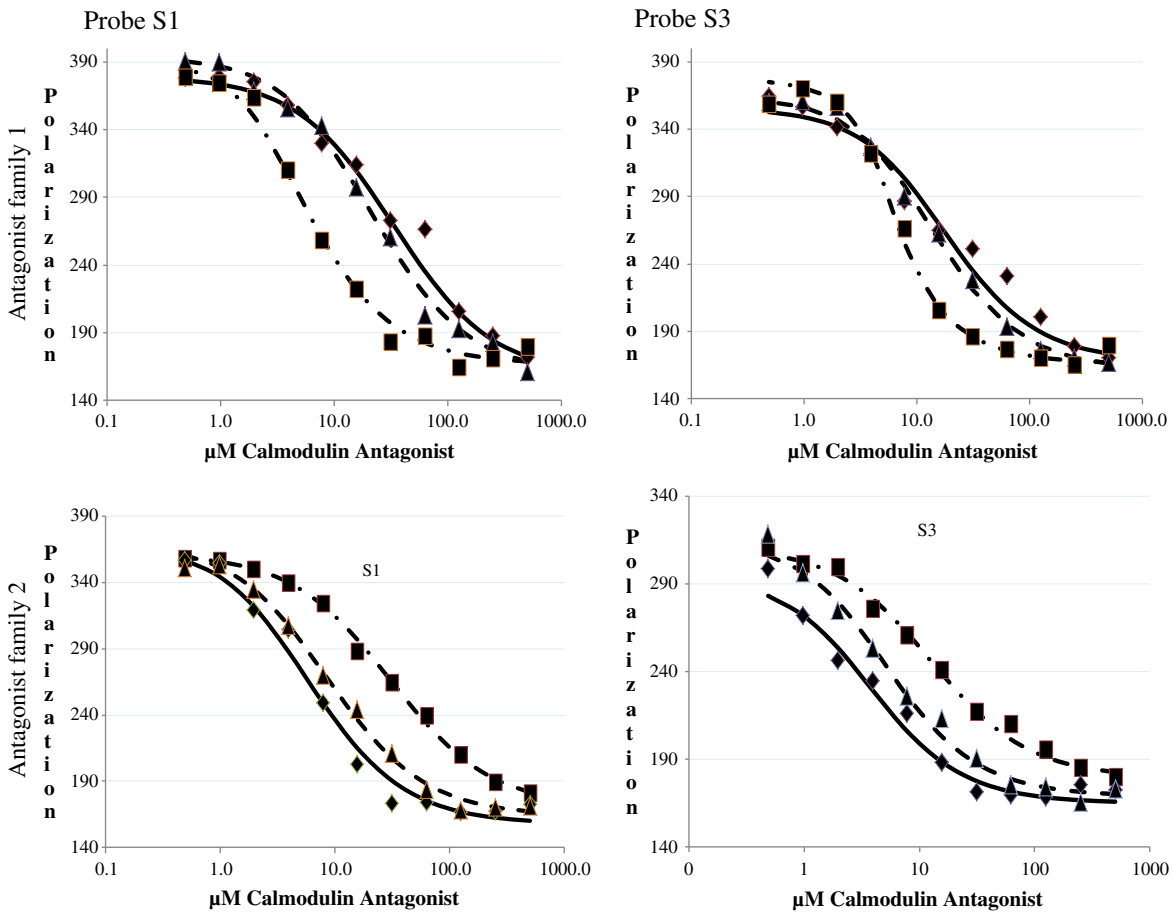

Fig. 3. Displacement of probes S1 and S3 by each of the selected CaM interactors. The six compounds presented in Table 1 and belonging to either family 1 (three compounds) or family 2 (three compounds) were tested for their potential to inhibit the binding to CaM of probe S1 (left panels) and probe S3 (right panels). Fluorescence polarization of the fluorescent probes S1 and S3 is followed as a function of CaM antagonists' concentration. Upper panel: family 1 CaM antagonists (■-•-■ stands for B05, ▲–▲for D02 and ♦—♦ for C08). Lower panel: family 2 CaM antagonists (■-•-■ stands for B10, ▲–▲ for D04 and ♦—♦ for C06). The data were fitted with a model of direct competition between the fluorescent probes S1 or S3 and the different CaM antagonists as described in [17]. CaM's concentrations range from 0.9 µM to 1.4 µM when used with probe S1 (0.1 µM) and from 2 to 4 µM when used with probe S3 (0.1 µM). Kd values for probe S1 and probe S3 binding to CaM are 0.16 µM and 0.89 µM, respectively. Ordinate correspond to polarization degrees P x 1000.

Ki values obtained by FP competition experiments (Tables 2 and 3). Ki values arising from FP studies are higher than the dissociation constants (Kd) obtained by calorimetry. The fact that the FP titration conditions differ from those used in the calorimetric studies by the presence, in the former, of 150 mM KCl points to a decrease of the overall affinity of the antagonists for CaM induced by the ionic strength.

Table 1
Parameters used to fit the data of Fig. 3 and residual mean square values for each fit. Methods used for data fitting are fully described in [17]. P_{max} is experimentally determined to be 420 (mP). Ki is the dissociation constant expressed in µM.

Molecules	Ki	P_{min}	RMS
Family 1			
LPS 02-10-L-D02_S1	2.02	159.12	62.43
LPS 02-10-L-D02_S3	2.52	161.33	41.76
LPS 02-10-L-C08_S1	4.85	157.80	62.63
LPS 02-10-L-C08_S3	4.07	167.47	100.84
LPS 02-20-L-B05_S1	0.48	166.79	65.81
LPS 02-20-L-B05_S3	0.46	165.89	50.12
Family 2			
LPS 01-18-L-B10_S1	6.75	170.39	21.79
LPS 01-18-L-B10_S3	5.40	179.37	34.90
LPS 01-18-L-D04_S1	1.53	163.79	42.70
LPS 01-18-L-D04_S3	1.79	168.77	43.66
LPS 01-18-L-C06_S1	1.02	157.81	76.62
LPS 01-18-L-C06_S3	1.56	164.87	61.62

3.4. Inhibition of the interaction between CaM and eight different CaM binding domains

In order to characterize the inhibitory potency of each of the six CaM antagonists, we selected 8 different CaM/Ca2 + binding domains and studied their interaction with CaM/Ca2 + in the absence and presence of the different antagonists. Each CaM binding domain is tagged with a fluorescent probe (TAMRA) as previously described [15] allowing the study of its interaction with CaM by FP. The sequences of the eight CaM binding domains (CBD) are given in Table 3. They correspond to:

- The CBDs of the plasma membrane calcium pump (PMCA) and the IQ domain of the L-type voltage dependent calcium channel (CaV),
- The CBDs of three CaM dependent kinases, namely the death associated protein kinase 2 (DAPK2), the smooth muscle myosin light chain kinase (smMLCK) and the skeletal myosin light chain kinase (skMLCK),
- Finally, the CBDs of three other CaM dependent enzymes, namely the CBD of the nitric oxide synthase of endothelial cell (eNOS), the CBD of the phosphatase PP3CA (calcineurin) and the CBD of the cAMP phosphodiesterase (PDE1A).

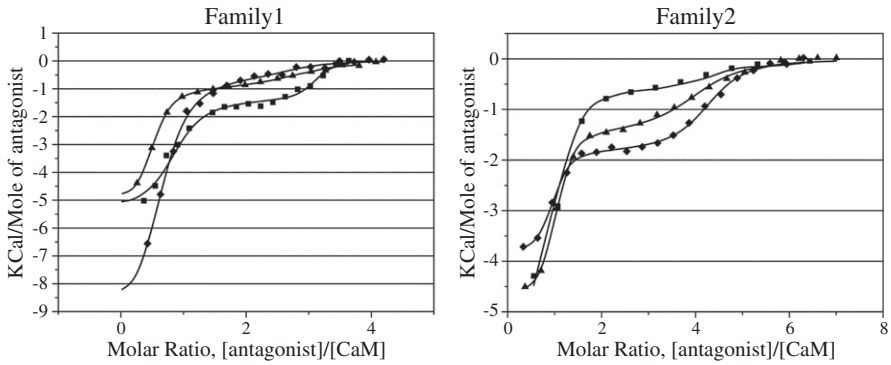

Fig. 4. Calorimetric study of antagonist binding to CaM. CaM was titrated by the CaM antagonists belonging to family 1 (left panel) and family 2 (right panel). Enthalpy changes (ordinate), expressed in kcal/mol of antagonist, are represented as a function of the antagonist/CaM molar ratio. For the molecules in family 1 (■-•-■ stands for B05, ▲–▲for D02 and ♦—♦ for C08); and in family 2 (■-•-■ stands for B10, ▲–▲for D04 and ♦—♦ for C06).

As the molecular weight of the different peptides is similar and the fluorescent probe is the same, the polarization must be the same if the peptide is in the monomeric form. The data presented in Table 3 indicate that all the peptides are in a monomeric form except the IQ domain of the voltage dependent calcium channel (CaV), the CaM binding domain of the plasma membrane calcium pump (PMCA) and DAPK2, which appears to associate in solution probably as dimers. The sequence of the CBD of DAPK2 is similar to the CBD sequences of the two other kinases (smMLCK and skMLCK) that are in a monomeric form. We may notice that DAPK2 is the only sequence to contain a cysteine residue and to have a leucine instead of a lysine in position 6.

The interaction of a fluorescently tagged CBD with CaM was monitored by recording its polarization as the CaM antagonists are added to the solution. The polarization is high in the absence of interactors. Addition of a given CaM antagonist lowers the polarization. CBD displacement is fitted using the Hill equation as presented in the Experimental section (Section 2.9).

3.4.1. Inhibition of the binding of the plasma membrane calcium pump and the L-type calcium channel CBDs to CaM

CaM in a calcium dependent manner activates the plasma calcium pump and in a similar manner facilitates or inactivates calcium entry through modulation of calcium channels. Therefore, CaM antagonists may modulate calcium homeostasis when applied to a given cell line [21]. In order to get more insight into this phenomenon, we focus in the present section on two CBDs involved in calcium signaling and modulation of calcium homeostasis. The displacement of the different CBDs from CaM as a function of CaM antagonist's concentration is presented in Fig. 5. The insets represent the relationship between the IC50 for a given CBD versus the Ki for each CaM antagonist. Two points can be noticed:

- In a given family, there is a reasonable exponential fit of the data,
- The two families appear to be uncorrelated. This suggests that the molecules of a given family bind to the same site with different affinities and the molecules of the two families bind to different sites on CaM.

Although the two families of molecules present similar affinities, the molecules of family 2 have a better inhibition potential for the calcium pump than the molecules of family 1 (the curve with the red squares on the right is compared to the curve with blue diamond). The opposite is seen with the calcium channel CBD. This suggests that the site where the CaM antagonist of a given family binds varies with the CaM/CBD complex. The present results suggest that it is preferable to use the molecules of family 2 to inhibit the plasma membrane calcium pump and the molecules of family 1 to inhibit the L-type calcium channels.

3.4.2. Inhibition of the binding to CaM of three CaM dependent protein kinase CBDs

CaM modulates the activity of a group of serine-kinases named CaM dependent kinases. We have previously analyzed the calcium dependency of the CBD of death associated protein kinase 1 (DAPK1) [15]. In this section of our study, we focus on three other CaM-dependent kinases, namely DAPK2 as well as the myosin light chain kinases from skeletal and smooth muscle.

The results are presented in Fig. 6 using the same format as in Fig. 5. For the two families of molecules and TFP, the inhibition potential is similar with IC50 in the 100 μM range. The molecules of each family

Table 2
Thermodynamic parameters of the six CaM antagonists. N corresponds to the number of binding sites, Ka to the association constants between CaM and each of the six antagonists, Kd to the dissociation constant (=1/Ka), ΔH is the change in enthalpy, ΔS is the change in entropy, and T is the temperature (°K). Ki is the mean dissociation constants between CaM and each molecule obtained from the fluorescence polarization (FP) experiments presented in Fig. 3.

Molecules	N	K_a, M^{-1}	K_d, nM	ΔH, kCal M^{-1}	TΔS, kCal M^{-1}	Ki μM
LPS 02-10-L-D02	0.4	3.20E+07	31.3	−5.3	4.9	2.27
	2.3	7.40E+05	1351.4	−1	7	
LPS 02-10-L-C08	0.6	2.90E+07	34.5	−9.5	0.7	4.46
	2.1	1.30E+06	769.2	−0.5	7.8	
LPS 02-20-L-B05	0.8	2.00E+08	5	−5.4	5.9	0.47
	2.2	7.00E+06	142.9	−1.4	8	
LPS 01-18-L-B10	0.9	3.90E+07	25.6	−4.8	5.6	6.07
	3.1	5.80E+05	1724.1	−0.7	7.2	
LPS 01-18-L-D04	0.9	6.30E+07	15.9	−4.7	5.9	1.66
	3	7.10E+05	1408.5	−1.5	6.5	
LPS 01-18-L-C06	0.8	1.30E+08	7.7	−3.9	7.1	1.29
	3.3	1.40E+06	714.3	−1.8	6.6	

Table 3
List and parameters of the CaM binding domains. Most of the peptides have been co-crystallized with CaM except the CBD of cAMP phosphodiesterase (PDE1A). Mw stands for the molecular weight of the peptide. Polarization corresponds to the value of the polarization degree (P x 1000) of the TAMRA-labeled peptide. In the column PDB, the name of the co-crystal in the protein data bank is reported [22–27].

Description	Sequence of the peptide	Position in the sequence	Mw	Name	Polarization	PDB
Ca2 + pump N-terminal domain	TAMRA-LRRGQILWFRGLNRIQTQIK-NH2	1123–1142	2907.5	PMCA	210	2KNE
Cardiac Ca(v)1.2; alpha-1C	TAMRA-KFYATFLIQEYFRKFKKRKEQ-NH2	1665–1685	3209.8	CaV	206	2F3Y
Death-associated protein kinase 2	TAMRA-RRRWKLSFSIVSLCNHLTR-NH2	312–330	2783.3	DAPK2	224	1WRZ
sk-Myosin light chain kinase	TAMRA-KRRWKKNFIAVSAANRFKKI-NH2	566–585	2872.5	smMLCK	160	2BBN
sm-Myosin light chain kinase	TAMRA-RRKWQKTGNAVRAIGRLSSM-NH2	1742–1761	2726.2	skMLCK	164	2K0F
Nitric-oxide synthase, endothelial	TAMRA-RKKTFKEVANAVKISASLMG-NH2	492–511	2589.1	eNOS	155	1NIW
Calcineurin	TAMRA-AAARKEVIRNKIRAIGKMARVFSVL-NH2	389–413	3208.9	Calcineurin	164	2R28
cAMP Phosphodiesterase	TAMRA-KPRFRSIVHVVQAGIFVERM-NH2	115–134	2780.3	PDE1A	165	ND

with the highest affinity inhibit the different CaM dependent kinases similarly. In this case, the two binding sites of each family are equally modified in the CBD–CaM complexes for the three CaM dependent kinases (see inset Fig. 6).

3.4.3. Inhibition of the binding of NO synthase, calcineurin and phosphodiesterase 1A CBDs to CaM

This section presents the interaction between CaM and the CBDs of nitric oxide synthase, the phosphatase calcineurin and cAMP phosphodiesterase 1A. Results are given in Fig. 7.

For NO synthase and calcineurin, the behavior of the two families of molecules is similar to the one observed for the CaM dependent kinases whereas for the phosphodiesterase, a slightly better inhibition is obtained with the molecules of the family 1.

Table 4 gathers the IC50s and the Hill coefficients used to fit the experimental data shown in Figs. 5 to 7. In each family of molecules and for all peptides, we observe a constant trend with $IC50_{C08} \sim IC50_{D02} > IC50_{B05}$ for the naphthalene derivatives and $IC50_{B10} > IC50_{D04} > IC50_{C06}$ for the aminopyridazine derivatives. However, this trend does not hold when considering the whole set of molecules with the whole set of CBDs. This suggests that we have at least two binding sites on CaM, one site for each chemical family. The family 1 molecules binding site (respectively the family 2 binding site) must be allosterically coupled to the binding sites of each CBD but in a different manner for each CBD peptide. Indeed, if it was not the case, for all CBDs, the two chemical families of molecules would behave the same way, meaning one set of molecules would always be a better inhibitor than the other set or the two sets would have the same potency for all CBDs.

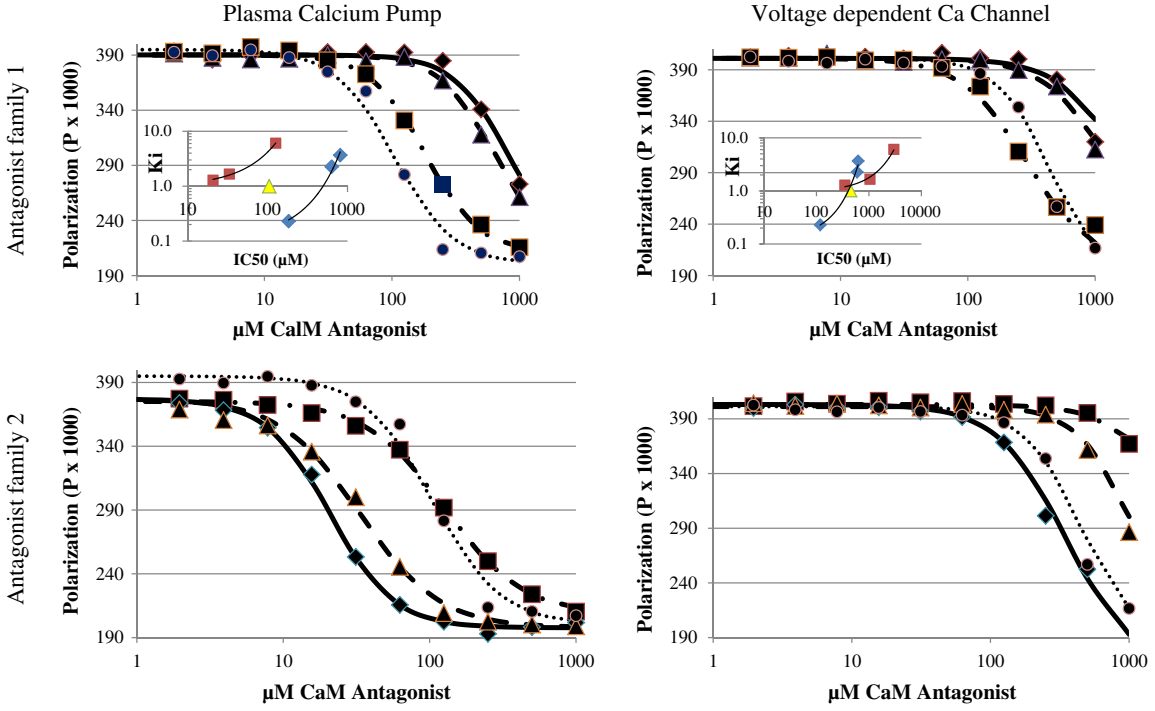

Fig. 5. Inhibition of the interaction between CaM and calcium binding domains (CBDs) by CaM antagonists. The interaction between CaM and the fluorescently labeled plasma calcium pump (left panels) and the L-type calcium channel (right panels) CBDs was monitored by fluorescence polarization (ordinate) as a function of the concentration of the six CaM antagonists belonging to two chemical families (see Fig. 1). Ordinate corresponds to the polarization degree P x 1000. Upper panel: chemical family 1 CaM antagonists (■-•-■ stands for B05, ▲–▲for D02 and ♦—♦ for C08). Lower panel: chemical family 2 CaM antagonists (■-•-■ stands for B10, ▲–▲for D04 and ♦—♦ for C06). The effect of trifluoroperazine (TFP) is represented in the four panels (●••●). Data were fitted with a phenomenological Hill model as described in section 2.9. CaM's concentrations ranged from 0.9 µM to 1.2 µM and the fluorescent peptides were set at 0.1 µM. The inset for each peptide represents the graph with IC50 obtained for each calmodulin antagonists (abscissa) and the value of Ki obtained from the competition experiment with probes S1 and S3 (red squares represent the family 2 molecules and blue diamonds the family 1 molecules). Represented Ki values correspond to the mean between the Ki values obtained with probes S1 and S3. Trifluoperazine is used as a control in this set of experiments (yellow triangles). Each experiment has been done at least twice. Error bars are not represented in the figures for sake of clarity but represent at most 3% of each experimental value.

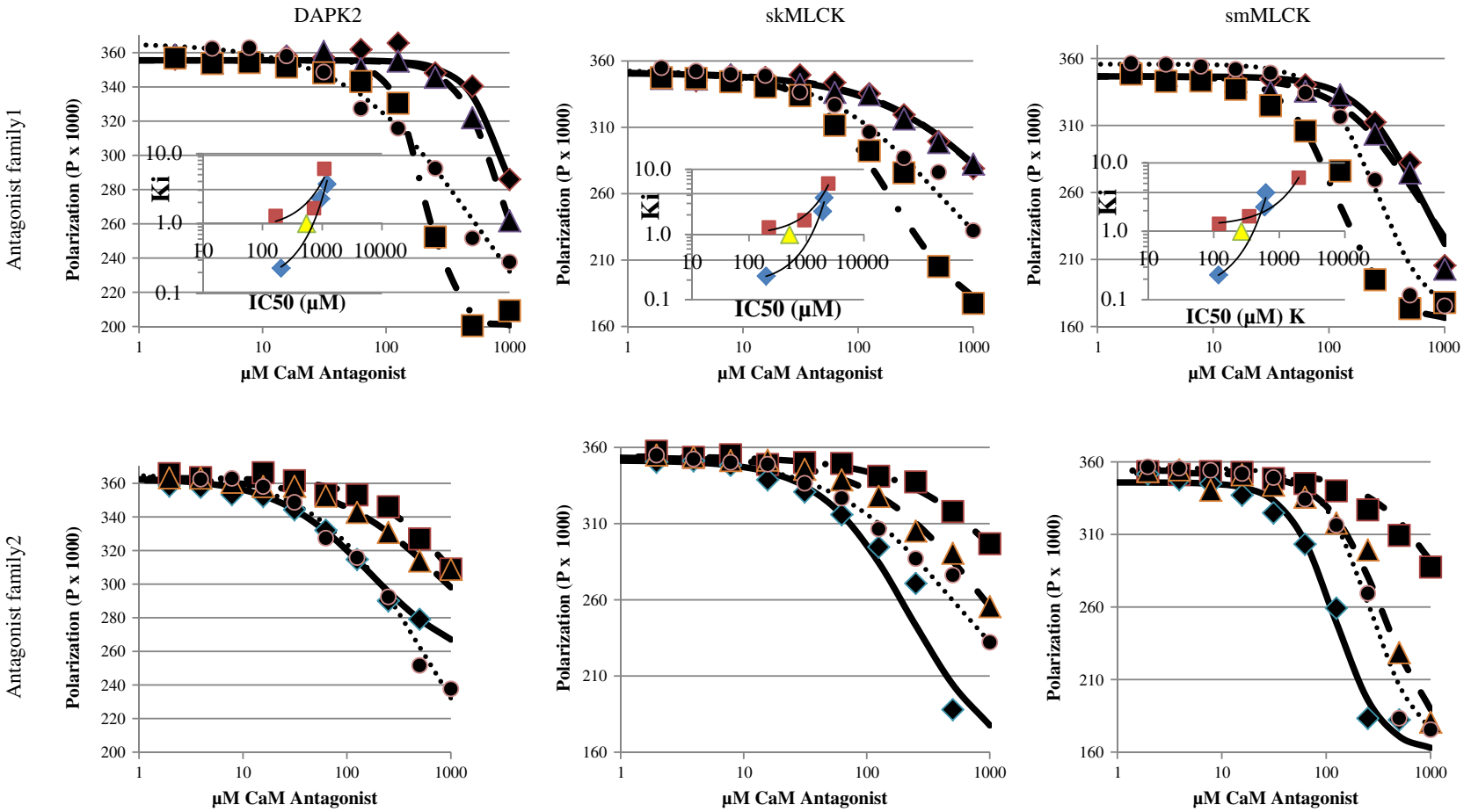

Fig. 6. Inhibition of the interaction between CaM and calcium binding domains (CBDs) by CaM antagonists. The interaction between CaM and the fluorescently labeled CBDs derived from the death associated protein kinase 2, the myosin light chain kinases from smooth muscle and the skeletal muscle by six CaM antagonists (Table 1). Fluorescence polarization of the fluorescent CBDs was followed upon titration of CaM by CaM antagonists belonging to chemical family 1 (upper panel) or family 2 (lower panel). Ordinate corresponds to the polarization degree P x 1000. Upper panel: family 1 CaM antagonists (■-•-■ stands for B05, ▲–▲ for D02 and ♦—♦ for C08). Lower panel: family 2 CaM antagonists (■-•-■ stands for B10, ▲–▲ for D04 and ♦—♦ for C06). The effect of trifluoroperazine (TFP) is represented in the four panels (●••●). Data were fitted with a phenomenological Hill model as described in section 2.9. CaM concentration ranged from 0.9 µM to 1.2 µM when used with 0.1 µM of fluorescent peptides. The inset for each peptide represents the graph with IC50 obtained for each CaM antagonists (abscissa) and the value of Ki obtained from the competition experiment with probes S1 and S3 (red square represents the family 2 molecules and blue diamond the family 1 molecules). Represented Ki values correspond to the mean between the Ki values obtained with probes S1 and S3. Trifluoperazine is used as a control in this set of experiments (yellow triangles). Each experiment has been done at least twice. Error bars are not represented in the figures for sake of clarity but represent at most 3% of each experimental value.

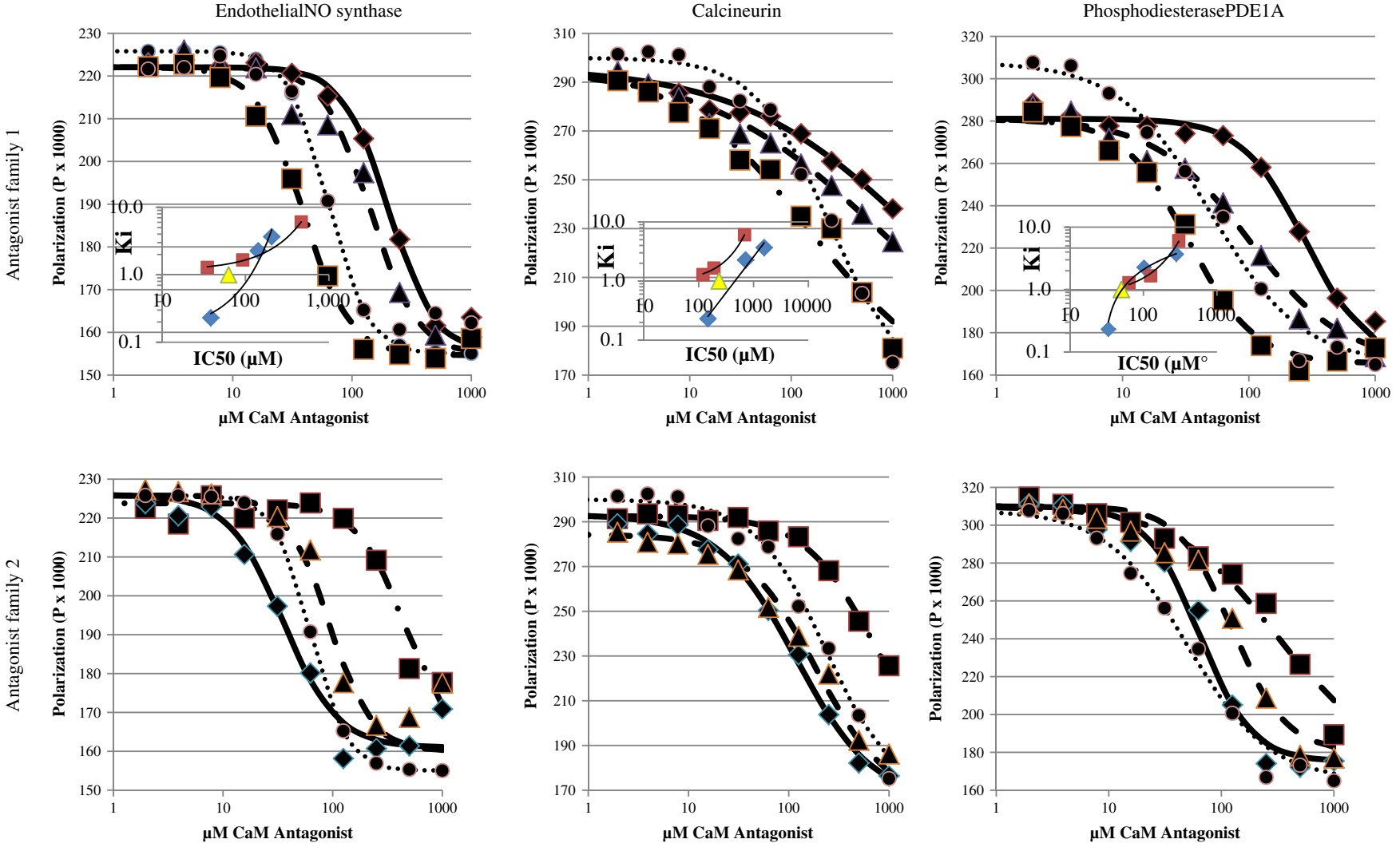

Fig. 7. Inhibition of the binding of three fluorescent tagged CaM binding peptides (the CBDs from endothelial NO synthase, cAMP phosphodiesterase 1A and calcineurin, a phosphatase) by six CaM antagonists belonging to the two families presented in Fig. 1. Fluorescence polarization of the fluorescent peptide (expressed as the fluorescence polarization degree P x 1000) was followed upon titration by CaM antagonists belonging to chemical family 1 (■-•-■ for B05, ▲–▲ for D02, ◆—◆ for C08) or to chemical family 2 (■-•-■ for B10, ▲–▲ for D04, ◆—◆ for C06) and by TFP (●••●). Data are fitted with a phenomenological Hill model as described in Section 2.9. CaM concentration ranged from 0.9 µM to 1.2 µM when used with fluorescent peptides at 0.1 µM. The inset for each peptide represents the graph with IC50 obtained for each CaM antagonists in abscissa and the value of Ki obtained from the competition experiment with probes S1 and S3 (red square represents the family 2 molecules and blue diamond the family 1 molecules). Represented Ki values correspond to the mean between the Ki values obtained with probes S1 and S3. Trifluoperazine is used as a control in this set of experiments ((yellow triangles). Each experiment has been done at least twice. Error bars are not represented in the figures for sake of clarity but represent at most 3% of each experimental value.

Table 4
IC50 values of each CaM antagonist for each CaM binding peptide obtained from experiments described in Figs. 5 to 7. IC50 is expressed in μM. The Hill coefficient of each curve is indicated between parentheses.

Molecules	PMCA	Cav	DAPK2	skMLCK	smMLCK	eNOS	Calcineurin	PDE1A
D02	633(2)	596(1.3)	940(2.3)	1908(.8)	596(1.3)	142(2.2)	714(.5)	102(1.2)
C08	809(2)	620(1.6)	1202(2.3)	2024(.8)	621(1.6)	206(2.2)	1562(.5)	279(1.7)
B05	183(2)	118(2)	204(3.4)	198(1.3)	118(2)	38(2.2)	148(.7)	33(1.7)
B10	126(1.6)	2981(1.8)	1082(1.1)	2396(.9)	1975(1.1)	466(2)	685(1.3)	302(1.0)
D04	33(1.6)	1045(1.8)	726(1)	919(.9)	347(1.6)	93(2.2)	189(1.2)	125(1.8)
C06	21(2)	347(1.8)	166(.9)	221(1.2)	120(1.9)	35(1.7)	119(1.1)	64(1.9)
TFP	105(1.9)	453(2)	543(.8)	506(.9)	264(1.9)	63(2.6)	236(1.2)	50(1.2)

Altogether, the two families of CaM antagonists can be used to get a typology of the CaM-Ca2+/CBD complexes. Three types of complex may be defined:

- The ones that are slightly better inhibited by the compounds of family 1 rather than those of family 2. The L-type calcium channel and PDE1A CBDs belong to this group. For these two CBDs, the molecule B05 has a better affinity than the molecule C06, although the affinity is weak for the L-type calcium channel CBD.
- The complexes where the two families of molecules exhibit a similar inhibition potential. This group is composed of the CBDs of the three CaM dependent kinases (DAPK2, MLCK from skeletal muscle and smooth muscle), of NO synthase and of calcineurin,
- The complexes where the molecules of family 2 inhibit the CaM/CBD complex better than the molecules of family 1. The CBDs of the plasma membrane calcium pump and cAMP phosphodiesterase 1A belong to this group.

4. Conclusion

The present study presents a general frame to detect CaM interactors in small molecule libraries and to characterize them. We have also set up a new strategy to analyze their ability to inhibit the interaction between CaM and different CaM binding proteins. Using a library composed of 5300 small molecules, two new chemical families of CaM antagonists were disclosed. In each family, three molecules exhibiting different affinities for CaM were characterized. These molecules pave the way for a more precise pharmacological study of CaM and its interaction with partners.

Moreover, to get more insight into the six selected CaM interactors, we have analyzed their potential to inhibit the interaction between CaM and a diverse set of CBDs. The inhibition potentials of the newly identified molecules were compared to those of the classical CaM antagonist, namely trifluoperazine (TFP).

This report shows the potential of small molecules to discriminate between different CaM/CBD complexes and thereby rejuvenates the pharmacology of CaM.

New screening tests using the fluorescently-tagged CBDs to detect molecules that inhibit a specific CaM complex may be set up. To be able to find molecules specific of a given CBD, it will be necessary to differentially screen small molecule libraries. Taking into account the number of proteins that interact with CaM and therefore, the number of CBDs, automation and miniaturization of the assay will be necessary. However, the effort is worth as CaM appears to be a hub protein in numerous important pathways within a cell and therefore, appears to be a strategic target to pinpoint in cancer and inflammatory diseases. The strategy presented in this report may be used to analyze the interaction between two proteins in a systematic way. Such a quest is becoming the grail of pharmaceutical companies.

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