A fluorescent polarization-based assay for the identification of disruptors of the RCAN1/calcineurin A protein complex.

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Short title: Towards the discovery of new immunosuppresants

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Abstract

Calcineurin is a Ca(2+)-calmodulin-dependent serine/threonine protein phosphatase involved in many biological processes and developmental programmes, including immune response. One of the most studied substrates of calcineurin is the transcription factors NFAT (Nuclear Factor of Activated T cell) responsible for T-cell activation. Different anticalcineurin drugs, such as cyclosporine A or FK506, are the most commonly immunosuppressants used in transplantation therapies. Unfortunately, their mechanism of action, blocking completely the calcineurin phosphatase activity together with the requirement of continuous administration, bears severe side effects. In the last years, the family of Regulators of Calcineurin (RCAN) has been described and extensively studied as modulators of calcineurin signalling pathways. The RCAN1-1 region, from amino acids 198 to 218, responsible for inhibiting *in vivo* the calcineurin-NFAT signaling pathway has been identified. An RCAN1-derived peptide spanning this sequence interferes with the calcineurin-NFAT interaction without affecting the general calcineurin phosphatase activity. We report here the development of an optimized *in vitro* high-throughput fluorescence polarization assay based on the disruption of the RCAN1$^{198-218}$-CnA interaction for identifying molecules with immunosuppressant potential. This approach led us to identify dipyridamole as a disruptor of such interaction. Moreover, three small molecules with a potential immunosuppressive effect were also identified.

Keywords: RCAN1, Calcineurin A, NFAT, immunosuppression, Fluorescence polarization assay, millipolarization units, combinatorial libraries.

Introduction

Calcineurin (Cn), a calcium and calmodulin dependent serine-threonine phosphatase, is an heterodimer formed by a catalytic subunit called calcineurin A (CnA) and a regulatory subunit called calcineurin B (CnB) (Klee 1979). This enzyme regulates several biological processes such as heart-valve morphogenesis, angiogenesis and neural and muscle development [2]. In human T cells, Cn dephosphorylates the cytoplasmic Nuclear Factors of Activated T cells (NFAT) [3] facilitating their translocation into the nucleus and consequently inititiating the immune response. Currently, immunosuppressive protocols used in transplantation and treatment of autoimmune diseases are based on the administration of calcineurin inhibitors, cyclosporine A (CsA) and FK506. Unfortunately, their mechanism of action, blocking completely the calcineurin phosphatase activity, together with the requirement of continuous administration, bears severe side effects. Thus, the identification of new and more selective molecules with lesser side effects than those currently in use is one of the main goals in the desired pharmacological control of immune response activation.

New methods that focus on recent discoveries on the molecular mechanism of Cn activity need to be devised in order to uncover modulators. In that sense, several endogenous Cn inhibitors such as the Regulators of Calcineurin family of proteins (RCAN, formerly known as DSCR1 or calcipressins) have been identified [8; 12]. Recently, our group has demonstrated the *in vivo* immunosuppressive role of human RCAN1 and RCAN3 proteins in human Jurkat T lymphocyte cells activated with ionomycin and phorbol 12-myristate 13-acetate (PMA) [13; 14]. In addition, we have reported on the identification and characterization of the RCAN amino acid sequence that inhibit Cn-NFAT signaling in activated Jurkat T cells, which in RCAN1 spans from amino acids 198 to 218 [1]. It is worth noting that a synthetic peptide derived from such region ($RCAN1^{198-218}$) interacts with CnA and inhibits the Cn-NFAT signalling without affecting general Cn protein phosphatase activity [1]. With these antecedents, an in vitro fluorescence polarization high-throughput screening assay based on the displacement of the interaction between CF-$RCAN1^{198-218}$ and CnA was developed leading to the identification of dipyridamole as a novel immunosuppressive molecule [1]. In the present study, we describe in detail such assay and the identification of three small molecules that significantly displace the $RCAN1^{198-218}$-CnA interaction.

Materials and methods

CnA large scale production

pGEX-6P-1-CnAα (human amino acids 2-347) plasmid construct was kindly provided by Dr. Patrick

Hogan [6]. BL21DE3 *E.coli* transformed with this plasmid was grown in Luria-Bertani medium at 37ºC 250 rpm. After reaching an optical density of 0.6-0.8 at 600 nm, GST-CnA production was induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich Co., St Louis, MO) over night at 25ºC. Then, cells were centrifuged at 5000xg 10 min at 4ºC, washed once in PBS supplemented with 0.5 mM PMSF (Sigma), and centrifuged again. Pellets were frozen immediately in dry ice and stored at –80ºC. These pellets were resuspended in a lysis buffer containing 0.5 mM DTT (Roche Diagnostics, IN), 0.2 mM $MgCl_2$, 1 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 mM PMSF, 10 μg/ml DNase I (Roche), 1mg/ml lysozime in PBS and incubated in a rotary shaker for 30 min at room temperature. Then, samples were sonicated, and after adding 1% triton X-100 (v/v), cells were incubated in a rotary shaker for 30 min at 4ºC. Finally, samples were subjected to three consecutive cycles of freezing at –80ºC and de-freezing in ice-water. After centrifugation of samples at 13.000xg for 30 min at 4ºC total bacterial protein extracts were obtained. Then, Glutathione Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) was used for purifying GST-CnA recombinant protein for 4 h at 4ºC following manufacturer's instruction. GST-CnA-Sepharose beads were collected after centrifugation and washed in a buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 mM PMSF in 50 mM Tris-HCl pH 7.0 and packed in a column. GST-CnA-Sepharose beads were washed twice with ten bed volumes of this buffer and eight times more with the same buffer without protein inhibitors. Then, GST-CnA-sepharose beads were incubated with the PreScission™ Protease (Amersham), which cleaves the specific protease cleavage site localized between the GST and CnA protein, in a rotary shaker over night at 4ºC. The purified CnA fractions were concentrated and quantified by spectrometry and protein integrity was confirmed by SDS-PAGE.

Peptide synthesis and molecules

The $RCAN1^{198-218}$ peptide, Ac-Lys-Tyr-Glu-Leu-His-Ala-Ala-Thr-Asp-Thr-Thr-Pro-Ser-Val-Val-Val-His-Val-Cys-Glu-Ser-NH_2, its N-terminal carboxyfluorescein (CF) derivative, the labeled CF-control peptide, CF-Gly-Gly-Met-Ala-Gly-Pro-His-Pro-Val-Ile-Val-Ile-Thr-Gly-Pro-His-Glu-Glu- NH_2 and the unlabeled control peptide, Ac-Ser-Ala-Val-Thr-His-Lys-Leu-Glu-Ser-Val-Asp-Pro-Ala-Thr-Val-Tyr-Cys-Glu-Thr-His-Val- NH_2. were synthesized as previously described [1]. MALDI-TOF mass spectrometry in a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) was used to confirm peptide identity. Several libraries of peptides and small molecules were screened to identify disruptor candidates of the CF-$RCAN1^{198-218}$-CnA interaction: a hexapeptide-based library, a diversity-oriented positional scanning library of *N*-alkylglycine trimers [15] and the Prestwick Chemical Library®.

All molecules diluted in 10% (v/v) DMSO in water were first analyzed in the assay at 100 μM. Only those compounds promoting a displacement of the CF-RCAN1$^{198-218}$-CnA interaction higher than 45% were further assayed to evaluate their effect in a dose-dependent manner in the same assay.

MALDI-TOF mass spectrometry

2.5 μM CnA was incubated in the absence or presence of 25 μM CF-RCAN1$^{198-218}$ peptide in PBS, in a final volume of 100 μl. Reactions were incubated for 15 min, 250 rpm at 25ºC. After adding 0.37% formaldehyde, samples were incubated 10 min more in the same assay conditions. Finally, reactions were stopped by adding 125 mM glycine. Samples were identified by MALDI-TOF mass spectrometry and data obtained were analyzed using the Data Explorer 4.5 software (Applied Biosystems).

Fluorescence polarization binding assay

The CF-RCAN1$^{198-218}$-CnA interaction was analyzed in black 96-well flat bottom plates (OptiPlate-96F(PB), Perkin Elmer) using a WallacVictor^2 1420 Multilabel HTS counter (PerkinElmer) and the Wallac 1420 Manager software. Parameters of the program used were as follows: excitation wavelength 480 nm, observed emission wavelength 535 nm, normal polarizer aperture, 0.1 s counting time and 1.2 Factor G Results obtained, indicated in millipolarization units (mP), were calculated with the following equation:

$$mP = 10^3 x \frac{(I_{pa} - 1.2 x I_{pe})}{(I_{pa} + 1.2 x I_{pe})}$$

I_{pa} and I_{pe}, correspond to the intensity of the paralel and perpendicular emitted light, respectively. The optimal binding protocol was obtained by adding 30 nM of CF-RCAN1$^{198-218}$ peptide to 200 μl of PBS (pH 7.4) containing increasing amounts of CnA for 15 min at room temperature. 0.01% (wt/vol) fraction V bovine serum albumin (BSA) (Roche) was added to the assay. To analyze the ability to displace the CF-RCAN1$^{198-218}$-CnA interaction, molecules were incubated for 15 min at room temperature with 1.5 μM CnA with the aim of facilitating their interaction, and then 30 nM CF-RCAN1$^{198-218}$ peptide was added to the assay. The final DMSO concentration in all the experiments was below 2% (v/v).

Results

The RCAN proteins play an immunosuppressive role in the human T lymphocyte Jurkat cell line by inhibiting the Cn-NFAT signalling pathway *in vivo* [13; 14]. As mentioned before, the identification of the RCAN1 sequence responsible for its biological role [1] tempted us to evaluate whether a RCAN1-derived peptide (RCAN1$^{198-218}$) spanning such amino acidic sequence could be a useful tool for identifying novel

molecules with immunosuppressant therapeutic potential. With this aim, we decided to set up and optimize an *in vitro* assay for analyzing the interaction between the RCAN1$^{198-218}$ peptide and CnA taking advantage of the fluorescence polarization technology.

First, we evaluated the behavior of a carboxifluorescein labelled RCAN1$^{198-218}$ peptide (CF-RCAN1$^{198-218}$) in 96-well plates in fluorescence polarization-based experiments in the absence of CnA (Fig. 1). We detected a high background when compared with a non-related carboxifluorescein labelled control peptide (Fig. 1, compare black bar in CF-RCAN1$^{198-218}$ peptide vs CF-control peptide). This result suggests that the CF-RCAN1$^{198-218}$ peptide has tendency to self-aggregate in solution or alternatively it becomes immobilized to the assay plates. To overcome this problem, we evaluated the efficiency of different blocking agents in the assay buffer. We tested two different reagents: 10% of immunoglobulin (IgG) and 1% of bovine fraction V serum albumin (BSA) (Figure 1, compare white bar and grey bar, corresponding to IgG and BSA, respectively, *vs* black bar, which represents absence of blocking agent). Our results show that adding blocking agents significantly reduces the background of the fluorescence polarization values obtained with the CF-RCAN1$^{198-218}$ peptide alone. Further characterization of the CF-RCAN1$^{198-218}$ peptide-CnA interaction was performed supplementing the assay with BSA.

In order to study the interaction between the CF-RCAN1$^{198-218}$ peptide and CnA we performed titration curves at a constant CF-RCAN1$^{198-218}$ peptide concentration (30 or 60 nM) and increasing CnA concentrations (in a range from 0.01 to 10 μM). In both cases, the CF-RCAN1$^{198-218}$-CnA interaction is established with similar efficiency and the polarization units increase in a CnA-dependent manner confirming the specificity of such interaction. Both conditions evaluated showed comparable millipolarization values (Fig. 2A). Thus, a 30 nM concentration of CF-RCAN1$^{198-218}$ peptide was routinely used in the assay. Furthermore, the actual binding of the RCAN1$^{198-218}$ peptide to CnA was also confirmed by peptide-protein crosslinking followed by mass spectrometry (MALDI-TOF) analysis (Fig. 2B). In the absence of the RCAN1$^{198-218}$ peptide or when CnA was incubated with the crosslinker agent and an unlabeled control peptide, the mass spectrum obtained showed only one peak corresponding to free CnA, which molecular mass is 41 kDa (Fig. 2B, see CnA and CnA-control peptide corresponding graphs). However, when CnA in presence of the RCAN1$^{198-218}$ peptide was crosslinked a second peak appeared in the mass spectrum, which total mass corresponds to the complex between RCAN1$^{198-218}$ peptide and CnA, which total molecular mass is 43 kDa (Fig. 2B, see RCAN1$^{198-218}$-CnA corresponding graph).

Next, we evaluated the steady-state optimal conditions for the CF-RCAN1$^{198-218}$-CnA interaction-

based assay. As depicted in Fig. 3A the millipolarization values obtained were constant after 15, 30 or 60 min at room temperature suggesting the formation of an stable complex after 15 min. The stabilization of a protein-protein or a peptide/protein interaction has a strong hydrophobic component arising from the interaction between the two large amino acidic surfaces provided. Then these complexes are sensitive to the presence of the organic solvents (DMSO is the preferred cosolvent) that usually contain the chemical libraries. Then, the DMSO concentration allowed by the CF-RCAN1$^{198-218}$-CnA interaction was analyzed and turned out to be very sensitive being the maximum DMSO concentration allowed at the assay only 2% (Fig 3B).

Once optimized all the parameters of the CF-RCAN1$^{198-218}$-CnA interaction in the *in vitro* fluorescence polarization assay, binding parameters for the CF-RCAN1$^{198-218}$ peptide were determined performing titration curves until reaching saturation. The results obtained showed that the K_d (dissociation constant) for the RCAN1$^{198-218}$ peptide is 1.25±0.09 μM and the IC_{50} is 3.72 ±0.28 μM[1]. This fact, together with the relative large millipolarization units increase shown (from 45-195 mP) and the small standard deviation obtained (less than 5%) has permitted us to develop a robust *in vitro* assay.

Different libraries composed by peptides or small molecules were used in order to identify molecules capable of disrupting the CF-RCAN1$^{198-218}$-CnA interaction, among them, the Prestwick Chemical Library®. This library includes 880 biologically active compounds with high chemical and pharmacological diversity. An initial screening assay leads us to identify some hit compounds that were also analyzed in a dose dependent manner to confirm their efficacy (Fig. 4). Table 1 summarizes the characteristics of the hit candidates analyzed. Further *in vivo* characterization of the most efficient molecule, dypiridamole, which is clinically used as an antiplatelet agent to prevent heart stroke, has revealed a previously unreported immunosuppressant role for this molecule [1]. Taken together, these results demonstrate that the *in vitro* assay here described is a useful tool for identifying novel molecules with *in vivo* immunosuppressive effect.

Discussion

Nowadays, immunosuppressive protocols used in transplantation and treatment of autoimmune diseases include always the use of calcineurin inhibitors due to the pivotal role of this enzyme in the activation of the immune response. Unfortunately, the multiple and severe side effects that their long-life administration produces, cancer, nephrotoxicity and neurotoxicity, makes necessary finding novel effective and less toxic agents. With this aim, Roehrl et al. developed an *in vitro* assay based on the interaction established between CnA and the VIVIT peptide [6], which inhibits NFAT signaling. The VIVIT

peptide amino acid sequence was selected from the natural SPRIEIT sequence on NFAT1 due to its high affinity binding to Cn [4]. Frim this assay, several immunosuppressant small molecules were identified in the Roehrl's assay, called INCA. Unfortunately, most of those molecules were reactive quinones that promoted severe cytotoxic effects to all evaluated cell Unfortunately, the in vivo use of these molecules in cell lines revealed a strongly high cytotoxicity due to their quinone structure [6]. Thus, we decided to direct our discovery efforts in finding new immunosuppresants towards the novel RCAN1-CnA interaction recently described [8]. Our results demonstrate that the RCAN1$^{198-218}$ peptide binds to CnA with high affinity and more importantly, this peptide inhibits Cn signaling towards NFAT, thereby preventing NFAT from being activated, without affecting general Cn phosphatase activity [1]. In this direction, we have recently shown that the RCAN1$^{198-218}$ peptide interfere with the SPRIEIT sequence on NFAT for binding to Cn. Thus, considering its biochemical properties together with its specific mechanism of inhibition towards the Cn-NFAT signalling, the RCAN1$^{198-218}$ peptide arises as an alternative and efficient tool for identifying novel immunosuppressive molecules. More importantly, its specific mechanism of inhibition towards the Cn-NFAT signalling indicates that the molecules identified might be also specific for the Cn-NFAT pathway *in cultutred T cells*.

With this aim, we set up and optimize the first *in vitro* fluorescence polarization-based assay targeting the interaction between the RCAN1$^{198-218}$ peptide and CnA. The results here presented indicate that this assay is specific, fast, highly reproducible, robust and could be easily standarized for high throughput screening analysis. As a consequence, it will increase the number of hit candidates that in turn will offer new possibilities aside from those molecules that promoted a total displacement of the Cn-NFAT interaction. Up to the present time, our group has evaluated dypiridamole as one compound identified from a chemical library using such an assay. The specific inhibitory capacity of dipiridamole obtained in *in vivo* experiments confirms the effectiveness of the assay in identifying novel immunossuppressive drugs [1]. Further structural analysis are ongoing to identify in detail the mechanism used by dipyridamole for disrupting the RCAN1-CnA interaction.

Furthermore, the data here presented increase the number of hit candidates with three new molecules with putative immunosuppressant role: coralyne chloride hydrate, daunorubicin hydrochloride and chicago sky blue 6B (Fig. 4). On one side, coralyne chloride hydrate and daunorubicin hydrochloride are drugs clinically used as antileukemic agents [16; 17]. On the other side, Chicago sky blue 6B is a potent inhibitor of L-glutamate uptake into synaptic vesicles [18] and recently it has been described that is an inhibitor of the Ca^{2+}/calmodulin-dependent protein kinase phosphatase family but not of calcineurin [19].

Thus, to date there is no evidence suggesting the existence of a novel immunossuppressive role for any of these three compounds. In a similar manner as it has been described for dipyridamole [1], we expect to further confirm their *in vivo* inhibitory effect towards the Cn-NFAT signalling pathway using cell lines as well as animal models.

Acknowledgments

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FIGURE LEGENDS

Table 1. Summary of the molecules with immunosuppressant potential responsible for disrupting the CF-RCAN1$^{198-218}$-CnA interaction identified from the Prestwick Chemical Library®

Fig. 1. Determination of the CF-RCAN1$^{198-218}$ peptide fluorescence background in the fluorescence polarization assay. The CF-RCAN1$^{198-218}$ peptide or a control non-related carboxyfluoresceinated peptide were incubated in the assay with no blocking agent (black bars) or in presence of two different blocking agents: 10% IgG (white bars) or 1% BSA (grey bars), respectively. The graphs show the mean ± SD of three independent experiments performed in triplicate.

Fig. 2. The CF-RCAN1$^{198-218}$ peptide interacts with CnA. **A.** Determination of the optimal CF-RCAN1$^{198-218}$ peptide concentration required to achieve the higher difference between the minimal and the maximum millipolarization values. Titration experiments were performed fixing the CF-RCAN1$^{198-218}$ peptide concentration (30 or 60 nM) in presence of increasing CnA concentrations (from 0.01 to 10 μM). Experiments were performed three times in triplicate. **B.** Evaluation of the RCAN1$^{198-218}$-CnA interaction by peptide-protein crosslinking followed by mass spectrometry (MALDI-TOF) analysis. Left panel corresponds to the assay including CnA alone; central panel corresponds to the assay including RCAN1$^{198-218}$ and CnA; right panel corresponds to the assay including an unlabeled RCAN non-related peptide together with CnA. CnA molecular mass is approximately 41 kDa; RCAN1$^{198-218}$ and Control peptides molecular mass is approximately 2.2 kDa.

Fig. 3. Characterization of the optimal assay conditions for the CF-RCAN1$^{198-218}$-CnA interaction. **A.** 15 min of incubation for a sample containing CF-RCAN1$^{198-218}$ and CnA is sufficient for the protein-protein interaction to take place. White bars correspond to free CF-RCAN1$^{198-218}$ peptide whereas black bars correspond to the CF-RCAN1$^{198-218}$-CnA established interaction. **B.** DMSO concentration above 2% affects the CF-RCAN1$^{198-218}$-CnA fluorescence polarization assay. White bars correspond to the free CF-RCAN1$^{198-218}$ peptide whereas black bars correspond to the CF-RCAN1$^{198-218}$-CnA. All experiments were performed twice in triplicate.

Fig. 4. Dose-dependent analysis of disruptors of the CF-RCAN1$^{198-218}$-CnA interaction identified from screening the Prestwick Chemical Library® by the fluorescence polarization assay. Data shown correspond to three independent experiments performed in triplicate.

TABLE 1

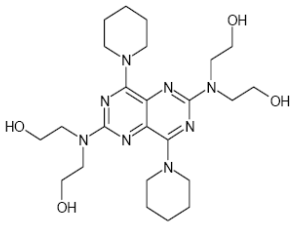
Table 1

Disruptor molecules of the RCAN$^{198-218}$-CnA interaction

1.- Dipyridamole

$C_{24}H_{40}N_8O_4$

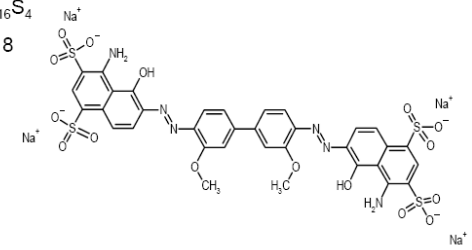
MW: 504,6376



2.- Chicago sky blue 6B

$C_{34}H_{24}N_6Na_4O_{16}S_4$

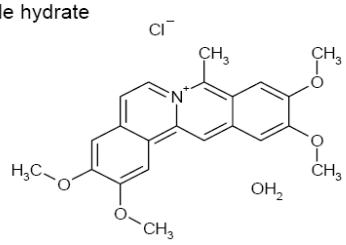
MW: 992,81618



7.- Coralyne chloride hydrate

$C_{22}H_{24}ClNO_5$

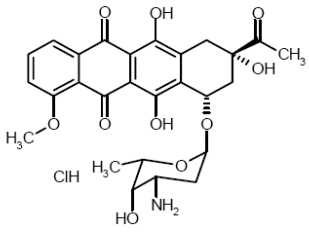
MW: 417,89328



8.- Daunorubicin hydrochloride

$C_{27}H_{30}ClNO_{10}$

MW: 563,99385



F

FIGURE 1

Figure 1

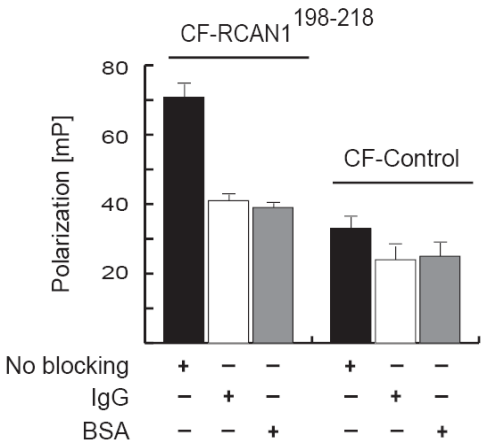


FIGURE 2

Figure 2

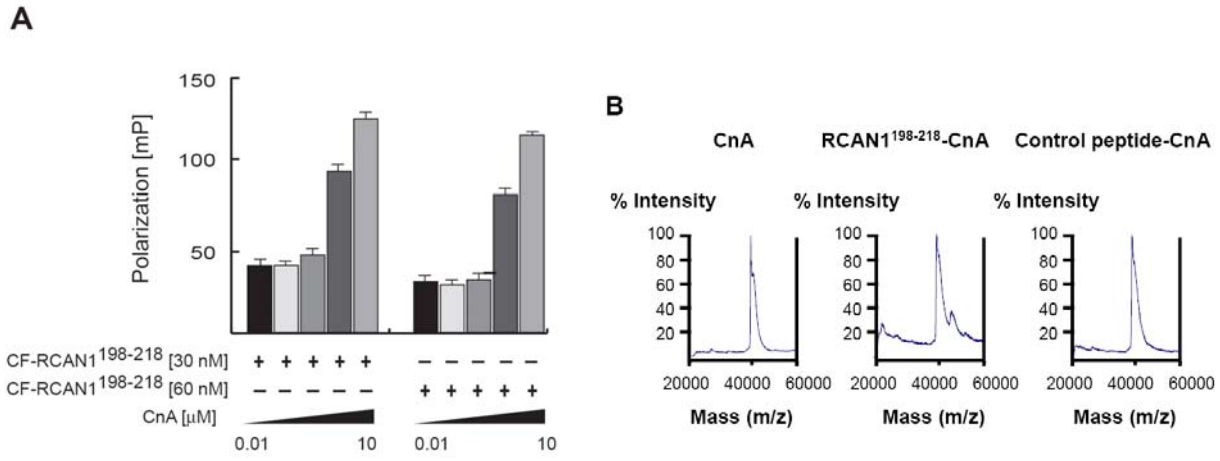


FIGURE 3

Figure 3

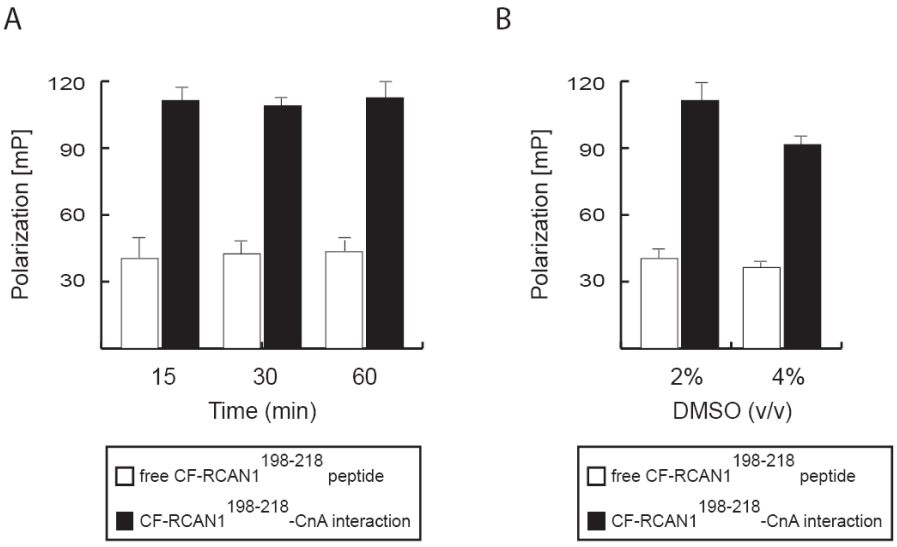


FIGURE 4

Figure 4