Switching feedback mechanisms realize the dual role of MCIP in the regulation of calcineurin activity

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Abstract  Calcineurin (CaN) assists T-cell activation, growth and differentiation of skeletal and cardiac myocytes, memory, and apoptosis. It also activates transcription of the nuclear factor of activated T-cells (NFAT) family including hypertrophic target genes. It has been reported that the modulatory calcineurin-interacting protein (MCIP) inhibits the CaN activity and thereby reduces the hypertrophic response. However, it has been shown that MCIP facilitates or permits the hypertrophic response under some stress conditions such as isoproterenol infusion or pressure overload by transverse aortic constriction. As there is no direct experimental evidence that can explain these paradoxical phenomena, there has been a controversy concerning the functional role of MCIP in developing the hypertrophic response. It is therefore crucial to establish a hypothesis that can clearly explain these phenomena. Towards this end, we propose in this paper a hypothesis that is based on available experimental evidence as well as mathematical modeling and computer simulations. We hypothesize that there is a threshold in the nuclear NFAT concentration above which MCIP is switched on. Below this threshold, the inhibition of active CaN by MCIP is negligible, while the activated protein kinase increases the dissociation rate of the CaN/MCIP complex. This leads to an augmentation of active CaN. This mechanism realizes the positive effect (i.e., removing any negative feedback) of MCIP in the hypertrophic response. On the other hand, the over-expression of active CaN increases nuclear NFAT to values above the threshold, while CaN is inhibited through binding of MCIP (expressed by the nuclear NFAT). This mechanism realizes the introduction of a negative feedback mechanism. To unravel this switching feedback mechanism, we have developed a mathematical model for which computer simulations are in agreement with the existing experimental data. The simulations demonstrate how the apparently paradoxical behavior can emerge as a result of cellular conditions.

1. Introduction

Calcineurin (CaN) is related to many physiological processes including T-cell activation, apoptosis, skeletal myocyte differentiation, and cardiac hypertrophy [1,12,17]. CaN enzyme is a heterodimer composed of calcineurin catalytic A subunit (CnA) and calcineurin regulatory B subunit (CnB). Its activity is suppressed through binding of C-terminal domain of CnA and autoinhibitory domain (AID) of the catalytic site at low Ca2+ concentration; however, as Ca2+ concentration increases, the protein phosphatase activity of CaN is induced by binding of Ca2+-bound calmodulin (CaM) since CaM binding displaces AID. There are three types of mammalian modulatory calcineurin-interacting protein (MCIP) genes: MCIP1, MCIP2, and MCIP3 [1]. Among these, MCIP1 and MCIP2 are predominant in cardiac myocytes, and only MCIP1 is activated by the active CaN (CaN*) [3,4]. MCIP2 is activated by thyroid hormone [3,4]. So, among the MCIP family, MCIP1 is the unique endogenous inhibitor of CaN and a well-conserved negative feedback loop component ranging from yeast to mammals [4,5]. In this respect, we focus only on MCIP1 among the MCIP family and simply use ‘MCIP’ instead of ‘MCIP1’ throughout the remaining part of this paper. MCIP binds with CaN and thereby inhibits the phosphatase activity of CaN. Intriguingly, some recent experiments have shown that MCIP can also facilitate or permit the CaN activity under some stress conditions such as isoproterenol (ISO) infusion or pressure overload (PO) by transverse aortic constriction (TAC) [2,6]. Such a role change of MCIP provides a good reason to consider it as a therapeutic target in cardiac hypertrophy.

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through regulation of the CaN-MCIP activity. Although the CaN signaling pathway receives a more attention than ever, the unknown underlying mechanism that realizes the dual role (role change) of MCIP in the CaN signaling pathway continues to pose important questions.

In this paper, we investigate available experimental results related to the controversy on the role of MCIP and based on this information develop a hypothesis that can explain the changing role of MCIP in the development of cardiac hypertrophy. In particular, we hypothesize that there is a threshold in the nuclear factor of activated T-cells (NFAT) concentration above which MCIP is switched on. We consider two different stress conditions, PO (or ISO infusion) and CaN* (over-expression of the active CaN expressed from a muscle specific transgene). Under the stress condition of PO, the concentration of nuclear NFAT (NFAT\textsubscript{nuc}) gets below the threshold and therefore the inhibition of CaN* by MCIP should be negligible; however, the release of CaN from the CaN/MCIP complex by some protein kinase (PK) increases CaN* so that NFAT\textsubscript{nuc} gets increased and incurs a hypertrophic response. This mechanism realizes the positive effect of MCIP in the development of cardiac hypertrophy by switching off the negative feedback. On the other hand, under the stress condition of CaN* (T), NFAT\textsubscript{nuc} gets above the threshold and therefore CaN* becomes inhibited by MCIP expressed from NFAT\textsubscript{nuc}. This inhibitory mechanism realizes the negative effect of MCIP by switching on the negative feedback. So, the functional role change of MCIP can be explained by this switching feedback mechanism. We present a mathematical model of the CaN-NFAT signaling pathway and quantitatively analyze the underlying dynamics and regulatory mechanism on the role change of MCIP through computer simulations. The simulation results are in agreement with existing experimental data and serve as an explanation for the role change of MCIP under different stress conditions.

2. Materials and methods

2.1. Experimental evidences on the functional role of MCIP

There has been a controversy about the functional role of MCIP in the development of cardiac hypertrophy caused by a series of recent experiments [2,5–7]. These experiments identified the role changing phenomena of MCIP under different stress conditions. We revisit previous experimental results and analyze their implications in order to infer a hypothesis that can explain the hidden mechanism of such paradoxical change of roles for MCIP.

Vega et al. [2] and Sanna et al. [6] showed that the hypertrophic response to CaN* over-expression was exacerbated in the MCIP1/−/− background (we refer to this experimental result as EXP 1 later on). Vega et al. showed that the increase of HW/BW (heart weight normalized to body weight) ratios caused by CaN* (T) was larger in the MCIP1/−/− animals than in the wild-type littermates (49% increase in CaN* (T) vs. 26% in WT). Sanna et al. more clearly showed Vega’s experimental results: the transgenic over-expression of the active CaN induced nearly 130% change of MCIP can be explained by this switching feedback mechanism. We present a mathematical model of the CaN-NFAT signaling pathway and quantitatively analyze the underlying dynamics and regulatory mechanism on the role change of MCIP through computer simulations. The simulation results are in agreement with existing experimental data and serve as an explanation for the role change of MCIP under different stress conditions.

2.2. Mathematical modeling of the CaN-NFAT signaling pathway

We develop a mathematical model of the CaN-NFAT signaling pathway, based on experimental evidence and analyze its dynamics to unravel the mechanism responsible for change of the functional role of MCIP. The detailed ordinary differential equations of the model are described in Table 2. CaN is allosterically activated from a free state ([CaN\textsubscript{free}]) to an active state ([CaN\textsubscript{act}]) through Ca²⁺/CaM binding while it is deactivated through a MCIP binding ([CaN\textsubscript{removed}]). These state transitions are described by Eqs. (1)–(3) in Table 2, where it is assumed that the active protein kinase (\(v_1\) in Eq. (2)) sigmoidally increases along with CaN* under stress conditions. CaN* ([CaN\textsubscript{removed}]) dephosphorylates NFAT\textsubscript{cyt} ([NFAT\textsubscript{ci}]) and NFAT\textsubscript{nuc} ([NFAT\textsubscript{nu}]) translocates into the nucleus to influence the transcription of hypertrophic genes, including

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>Mutation</th>
<th>Heart size compared with a wild-type littermate</th>
<th>Experimental number</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stress</td>
<td>MCIP+/+</td>
<td>No change</td>
<td>EXP 2, EXP 4</td>
<td></td>
</tr>
<tr>
<td>PO, ISO Infusion</td>
<td>MCIP+/−</td>
<td>Slight increase</td>
<td>EXP 1, EXP 3</td>
<td></td>
</tr>
<tr>
<td>CaN* (T)</td>
<td>MCIP+/−</td>
<td>Increase</td>
<td>EXP 1, EXP 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCIP+/+</td>
<td>Increase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The hypertrophic response is graded as ‘no change’, ‘slight increase’, ‘increase’, and ‘severe increase’ according to the order of HW/BW.
Fig. 1. Illustration of the hypertrophic response to different stress stimuli reported in the previous experiments [2,6]. The blue solid line and the red dotted line represent the hypertrophic response of MCIP1−/− TG and the wild-type, respectively. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

ing MCIP (\(MCIP\)). On the other hand, protein kinases such as glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\), p38, and cAMP dependent protein kinase A (PKA), represented by GPP (\(GPP\)), phosphorylate NFAT\(_{cyt}\) and antagonizes CaN by stimulating the nuclear NFAT export showing a potential antihypertrophic activity [16]. The regulatory relationships of NFAT and GPP are described by Eqs. (4)–(6) in Table 2. The expression of MCIP induced by NFAT\(_{nuc}\) (\([\text{NFAT}_{nuc}]\)) is described by Eq. (7) in Table 2. All the parameter values of the mathematical model are summarized in Table 3.

To investigate the dynamics of the CaN-NFAT signaling pathway and the underlying role change mechanism of MCIP under different stress conditions, we have conducted extensive computer simulations over a broad range of Ca\(^{2+}\) concentrations (10 nM to 1 \(\mu\)M) as the Ca\(^{2+}\) levels represent different stress conditions [19–22]. The range of Ca\(^{2+}\) concentrations was chosen based on the experimental evidence presented in [19–22]. For instance, the resting Ca\(^{2+}\) concentration of cardiac myocytes is less than 0.2 \(\mu\)M under a normal condition, but it becomes more than 0.7 \(\mu\)M under a stress condition in mice, allowing for some variations related to the species (e.g., rat, ferret, guinea pig, canine, etc.). Throughout the simulations, we assumed a constant Ca\(^{2+}\) concentration since CaN responds to sustained, low frequency Ca\(^{2+}\) [18].

Let us define a measure called area under curve (AUC) of NFAT\(_{nuc}\) to quantify the hypertrophic response to Ca\(^{2+}\) stimulation as follows:

\[
\text{AUC}(t, \text{Ca}^{2+}) = \int_{0}^{t} \text{NFAT}_{nuc}(\tau, \text{Ca}^{2+}) \, d\tau
\]

In the simulations, we cover an interval to \(t = 20\, \text{h}\) since NFAT\(_{nuc}\) reaches its steady-state value after about 20 h, under different Ca\(^{2+}\) stimulations (Fig. 6). The equations in Table 2 were solved using the Runge–Kutta–Merson numerical integration algorithm with Matlab 7.1 (R14) on an HP workstation xw6000.

3. Results

In this section, we present a hypothesis on the role changing mechanism of MCIP, based on the previously published experimental results (EXP 1–7) and demonstrate the dynamical characteristics of this mechanism under different stress conditions through computer simulations of the proposed mathematical model.

3.1. The switching feedback mechanism under different stress conditions

The \(\beta\)-adrenergic signaling pathway is activated by a variety of stress stimuli including PO and \(\beta\)-AR agonist. If then, the intracellular Ca\(^{2+}\) becomes increased by the downstream proteins such as PKA and Ca\(^{2+}\)/calmodulin dependent protein kinase II (CaMKII); CaN is activated by Ca\(^{2+}\)/CaM. Finally, NFAT is dephosphorylated by Ca\(^{2+}\) and then translocates into the nucleus. It eventually induces the transcription of a variety of genes involved in the response of cardiac hypertrophy [2,9,10]. It has been assumed that the hypertrophic response gets enhanced along with the concentration of Ca\(^{2+}\) [2,6,23]. Since MCIP−/− mice exhibited no overt phenotype in the absence of stress [2], we presume that the concentration of Ca\(^{2+}\) in MCIP−/− mice is almost same as that of WT.

### Table 2

The mathematical model of the CaN-NFAT signaling pathway

\[
\frac{d[CaN_{nuc}]}{dt} = -k_{a1} \cdot [CaN_{nuc}] \cdot [Ca^{2+}] + k_{a2} \cdot [CaN_{active}]
\]

(1)

\[
\frac{d[CaN_{comp}]}{dt} = k_{a2} \cdot [CaN_{active}] \cdot \frac{[MCIP]^{1+k_2}}{k_{a2} + [MCIP]^{2}} - k_{a2} \cdot \gamma \cdot [CaN_{comp}]
\]

where \(\gamma = \frac{[CaN_{active}]^{k_2}}{k_{a2}^{k_2} + [CaN_{active}]^{k_2}}\)

(2)

\[\begin{align*}
[CaN_{act}] &= [CaN_{nuc}] + [CaN_{active}] + [CaN_{comp}] \\
[\text{NFAT}_{cyt}] &= \frac{k_1 \cdot [\text{NFAT}_{nuc}] \cdot [CaN_{active}]^{k_2}}{k_{a2} + [CaN_{active}]^{k_2}} - (l_{ext} + k_{e1}) \cdot [\text{NFAT}_{cyt}] + [\text{NFAT}_{nuc}] \\
[\text{NFAT}_{nuc}] &= l_{ext} \cdot [\text{NFAT}_{cyt}] - l_{inc} \cdot [\text{NFAT}_{nuc}] - \frac{k_{e2} \cdot [GPP] \cdot [\text{NFAT}_{nuc}]}{k_{a2} + [\text{NFAT}_{nuc}]} \\
[\text{MCIP}] &= \frac{k_{e1} \cdot [\text{NFAT}_{nuc}] \cdot [\text{cof}]^{k_2}}{k_{e2} + [\text{NFAT}_{nuc}] + [\text{cof}]^{k_2}}
\end{align*}\]

Note that the protein kinases GSK-3\(\beta\), p38, and PKA that phosphorylate NFAT\(_{cyt}\) in Fig. 2 are represented by GPP in the equations.
Table 3
Parameters of the mathematical model used for simulations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Unit</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{d1}$</td>
<td>0.3</td>
<td>$\mu$M$^{-1}$ min$^{-1}$</td>
<td>Association rate constant of Ca$^{2+}$ and CaN</td>
</tr>
<tr>
<td>$k_{d2}$</td>
<td>0.08</td>
<td>min$^{-1}$</td>
<td>Dissociation rate constant from the Ca$^{2+}$/CaN complex [13]</td>
</tr>
<tr>
<td>$k_{d3}$</td>
<td>8.53</td>
<td>$\mu$M$^{-1}$ min$^{-1}$</td>
<td>Association rate constant of CaN and MCIP</td>
</tr>
<tr>
<td>$k_{d4}$</td>
<td>5.67e$^{-2}$</td>
<td>min$^{-1}$</td>
<td>Dissociation rate constant from the CaN/MCIP complex</td>
</tr>
<tr>
<td>$k_{1}$</td>
<td>2</td>
<td>$\mu$M$^{-1}$ min$^{-1}$</td>
<td>Dephosphorylation rate constant of NFAT by CaN$^*$ [13]</td>
</tr>
<tr>
<td>$k_{iso}$</td>
<td>0.4</td>
<td>$\mu$M</td>
<td>Half maximal concentration of CaN</td>
</tr>
<tr>
<td>$k_{p1}$</td>
<td>4.5e$^{-4}$</td>
<td>$\mu$M$^{-1}$ min$^{-1}$</td>
<td>NFAT phosphorylation rate constant of GPP</td>
</tr>
<tr>
<td>$k_{p2}$</td>
<td>0.01</td>
<td>$\mu$M</td>
<td>Half maximal concentration of GPP</td>
</tr>
<tr>
<td>$t_1$</td>
<td>0.02</td>
<td>min$^{-1}$</td>
<td>MCIP expression rate constant [13]</td>
</tr>
<tr>
<td>$k_{nc}$</td>
<td>0.03</td>
<td>$\mu$M</td>
<td>Half maximal concentration of NFAT$_{nuc}$</td>
</tr>
<tr>
<td>$l_{nu}$</td>
<td>5.4e$^{-3}$</td>
<td>min$^{-1}$</td>
<td>Import rate constant of NFAT from cytoplasm to nucleus</td>
</tr>
<tr>
<td>$l_{nu}$</td>
<td>4.8e$^{-4}$</td>
<td>min$^{-1}$</td>
<td>Export rate constant of NFAT from nucleus to cytoplasm [14]</td>
</tr>
<tr>
<td>$k_{p3}$</td>
<td>0.001</td>
<td>$\mu$M</td>
<td>Half maximal concentration of active CaN</td>
</tr>
<tr>
<td>$k_{p4}$</td>
<td>0.005</td>
<td>min$^{-1}$</td>
<td>Constitutive phosphorylation rate of NFAT</td>
</tr>
<tr>
<td>$k_{deg}$</td>
<td>0.1</td>
<td>min$^{-1}$</td>
<td>Degradation rate of MCIP</td>
</tr>
<tr>
<td>$h$</td>
<td>17</td>
<td>–</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>$h_1$</td>
<td>5</td>
<td>–</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>$h_2$</td>
<td>15</td>
<td>–</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>cof</td>
<td>0.025</td>
<td>$\mu$M</td>
<td>Cofactor for NFAT transcription</td>
</tr>
<tr>
<td>$k_{th}$</td>
<td>0.037</td>
<td>$\mu$M</td>
<td>Threshold value of NFAT$_{nuc}$</td>
</tr>
<tr>
<td>GPP</td>
<td>0.01</td>
<td>$\mu$M</td>
<td>Concentration of GPP</td>
</tr>
<tr>
<td>CaN$_{tot}$</td>
<td>1.0</td>
<td>$\mu$M</td>
<td>Total concentration of CaN [15]</td>
</tr>
<tr>
<td>NFAT$_{tot}$</td>
<td>0.017</td>
<td>$\mu$M</td>
<td>Total concentration of NFAT [13]</td>
</tr>
</tbody>
</table>

Hence, we can identify the role of MCIP by comparing the experimental results under different stress stimuli which alters the concentration of CaN$^*$ in MCIP$^{+/–}$ mice and WT. The hypertrophic response under PO or ISO infusion occurs more significantly in WT than in MCIP$^{+/–}$ mice [EXP 2]. The MAP kinase signaling pathway is triggered by the activated β-AR and epidermal growth factor receptor (EGFR) [6,11] and then its downstream signaling proteins such as mitogen-activated protein kinase 5 (MEK5) and big mitogen-activated protein kinase 1 (BMK1) are activated. These protein kinases induce the phosphorylation of MCIP and thereby CaN is released from the CaN/MCIP complex [EXP 7]. MCIP in WT is, however, not increased enough to suppress the activity of CaN in response to PO or ISO infusion [EXP 6]. So, we infer that the concentration of NFAT$_{nuc}$ under PO or ISO infusion is not sufficiently increased to induce MCIP. On the other hand, MCIP inhibits the activity of CaN through binding with its catalytic domain in vivo and in vitro [EXP 5]. Under the stress condition of CaN$^*$, the hypertrophic response gets blunt in WT compared to that of MCIP$^{+/–}$ mice [EXP 1]. These imply that NFAT$_{nuc}$ under this stress condition increases MCIP such that it suppresses CaN$^*$. Thus, we infer that there is a threshold in the concentration of NFAT$_{nuc}$ by which MCIP is turned on or off depending on the stress conditions. By integrating these inferences, we propose a hypothesis on the role changing mechanism of MCIP as follows: under PO or ISO infusion, the expression of MCIP is negligible since the concentration of NFAT$_{nuc}$ is below the threshold and therefore CaN$^*$ is hardly inhibited by MCIP. In addition, the dissociation of the CaN/MCIP complex becomes accelerated by the activated protein kinase and this increases CaN$^*$ leading to a hypertrophic response. Hence, this mechanism realizes the positive effect (i.e., switching off the negative feedback) of MCIP in the hypertrophic response. On the other hand, under CaN$^*$ condition, NFAT$_{nuc}$ gets increased above the threshold and this makes CaN$^*$ significantly inhibited by MCIP. Thus, this feedback mechanism realizes the negative effect (i.e., switching on the negative feedback) of MCIP. Although CaN can be released from the CaN/MCIP complex by the protein kinase, the amount is negligible compared to that inhibited by MCIP. In summary, we conclude that the switching feedback mechanism of the negative feedback loop realizes the dual role of MCIP in the regulation of the CaN activity (see Fig. 2).

3.2. The simulation results

The system responses under two typical stress conditions are shown in Fig. 3 (0.4 $\mu$M of Ca$^{2+}$ for a strong stress condition) and Fig. 4 (0.04 $\mu$M of Ca$^{2+}$ for a mild stress condition). In Figs. 3 and 4, the panels A and B and the panels C and D show the system dynamics of WT and MCIP$^{+/–}$, respectively. Throughout this simulation study, we analyze the changing feature of hypertrophic response and the underlying MCIP regulatory mechanism given the typical stress conditions. In WT mice under the strong stress condition (Fig. 3A and B), it takes ca. 35 min after Ca$^{2+}$ stimulation for CaN$^*$ to reach its maximum value of 0.35 $\mu$M and then CaN$^*$ gets progressively decreased to its steady-state value of 0.1 $\mu$M in 2.6 h as shown in Fig. 3A. In the meanwhile, MCIP keeps a small concentration level in the initial phase and gets increased after 2.4 h. MCIP reaches its maximum value of 0.154 $\mu$M in 3.7 h (Fig. 3A). Hence, it turns out that MCIP effectively suppresses CaN$^*$ through the negative feedback mechanism under the strong stress condition. On the other hand, NFAT$_{cyt}$ gets increased to its maximum value of 16 nM in 18 min as the increase of CaN$^*$ in the initial phase induces the rapid decrease of NFAT$_{cyt}$ (Fig. 3B). However, after 1.2 h, NFAT$_{cyt}$ increases since CaN$^*$ gets inhibited by MCIP and this induces the phosphorylation of NFAT$_{cyt}$. NFAT$_{cyt}$ increases up to 12.8 nM in 9.3 h due to the sustained inhibition of CaN$^*$ by MCIP. Thus, NFAT$_{cyt}$ gradually decreases after 18 min and finally reaches its minimum value in 7.5 h. In inverse proportion to NFAT$_{cyt}$, NFAT$_{nuc}$ increases to its maximum value of 7.27 nM in 3.25 h;
however, it gets progressively decreased and reaches its steady-state value of 3.5 nM in 10 h. All these simulations are in agreement with previously published experimental results and provide a good rationale in understanding the functional role of MCIP in Exp 1 and Exp 3 [2,6].

In MCIP/−/− mice under the strong stress condition (Fig. 3C and D), CaN* monotonically increases to the steady-state value of 0.3 μM in 32 min after Ca²⁺ stimulation (Fig. 3C). Unlike CaN* in WT, CaN* in MCIP/−/− mice does not decrease since the CaN inhibitor, MCIP is completely deleted. On the other hand, NFAT cyt rapidly increases to its maximum value of 15.6 nM in 21 min due to the increase of CaN* in the initial phase; however, NFAT cyt gets gradually decreased to its steady-state value of 5.5 nM which is larger than that of WT.

Fig. 2. A schematic diagram illustrating the CaN-NFAT signaling pathway and its regulatory mechanisms.

Fig. 3. Computational simulations of the CaN-NFAT signaling pathway under a strong stress condition (0.4 μM of Ca²⁺). The active CaN gets much suppressed (A) and the nuclear NFAT is attenuated (B) as MCIP increases in WT. The active CaN and the nuclear NFAT monotonically increase for MCIP/−/− (C and D).
(2.8 nM) since CaN* in MCIP−/− mice is larger than that of WT. In inverse proportion to NFAT cyt, NFAT nuc increases to its maximum value of 11.4 nM (Fig. 3D) which is 226% larger than that of WT. From these simulation results, we found that the hypertrophic response represented by NFAT nuc becomes more significantly augmented in MCIP−/− than in WT under the strong stress condition, which is in agreement with the experimental evidence presented in Exp 1 and Exp 3 [2,6].

In WT mice under the mild stress condition (Fig. 4A and B), CaN* increases to its maximum value of 0.123 μM in 2.2 h after Ca²⁺ stimulation and then progressively decreases (Fig. 4A). This maximum CaN* is lower than that of the strong stress condition in Fig. 3A. This is because Ca²⁺ stimulation increases along with the strength of stress. The attenuation of CaN⁺ by MCIP is remarkably reduced since the expression of the CaN inhibitor, MCIP is very small compared to that of the strong stress condition. On the other hand, NFAT cyt gets increased to its maximum value of 5.15 nM in 2.8 h after Ca²⁺ stimulation and then converges to its steady-state value of 1.61 nM (Fig. 4B). However, the maximum value of NFAT cyt is much smaller and the rising time to the maximum value is also more prolonged compared to those under the strong stress conditions due to the reduced CaN⁺. Hence, NFAT nuc is significantly attenuated with the 108% decreased maximum value (3.49 nM in 5.32 h) compared to that of the strong stress condition (Fig. 4B). The rising time taken for NFAT nuc to reach its maximum value is prolonged by 64% compared to that of the

Fig. 4. Computational simulations of the CaN-NFAT signaling pathway under a mild stress condition (0.04 μM of Ca²⁺). The active CaN in WT (A) is larger than that of MCIP−/− (C) and the nuclear NFAT in WT (B) is also larger than that of MCIP−/− (D).

Fig. 5. The AUC of nuclear NFAT according to the increase of Ca²⁺ stimulation in WT and MCIP−/− (A), and the crossing point between WT and MCIP−/− in (A) along with the initial CaN/MCIP complex concentration (B). The AUC curves are in agreement with the previous experimental results shown in Fig. 1.
strong stress condition (3.25 h). Consequently, the expression of MCIP induced by NFAT_{nuc} is significantly suppressed under the mild stress condition. This simulation result is in agreement with EXP 6 [6] in that MCIP does not much increase under the mild stress condition such as PO or ISO infusion.

In MCIP\textsuperscript{+/−} mice under the mild stress condition (Fig. 4C and D), CaN\textsuperscript{+} increases to its steady-state value of 65 nM in 1 h after Ca\textsuperscript{2+} stimulation (Fig. 4C), but NFAT\textsubscript{cyc} and NFAT\textsubscript{nuc} are little increased (Fig. 4D) since the total concentration of CaN is decreased by 50\% in MCIP\textsuperscript{+/−} compared to that of WT.

Fig. 5A shows the AUC profiles according to the variation of Ca\textsuperscript{2+} concentration in MCIP\textsuperscript{+/−} and WT models where the initial concentration of the CaN/MCIP complex ([CaN\textsubscript{comp}(0)]) is 0.5 \(\mu\)M. Note that the crossing point between the two AUC curves of MCIP\textsuperscript{+/−} and WT in Fig. 5A delimits the functional role of MCIP as follows: if Ca\textsuperscript{2+} < 0.08 \(\mu\)M, MCIP has the positive effect (i.e., switching off the negative feedback) in that the hypertrophic response in WT is larger than that of MCIP\textsuperscript{+/−}; if Ca\textsuperscript{2+} \(\geq\) 0.08 \(\mu\)M, it has the negative effect (i.e., switching on the negative feedback) in that the hypertrophic response in WT is smaller than that of MCIP\textsuperscript{+/−}. The Ca\textsuperscript{2+} concentration at the crossing point gets increased along with [CaN\textsubscript{comp}(0)] (Fig. 5B). In particular, above 0.5 \(\mu\)M, the crossing point increases in a non-linear way. These results suggest that the negative effect of MCIP
emerges at a higher Ca\(^{2+}\) concentration (for a stronger stress condition) as [Ca\(N_{\text{comp}}^{{(0)}}\)] becomes larger. For instance, if [Ca\(N_{\text{comp}}^{{(0)}}\) = 0.5 \(\mu\)M, the negative effect of MCIP emerges at 0.09 \(\mu\)M Ca\(^{2+}\) while, if [Ca\(N_{\text{comp}}^{{(0)}}\) = 0.7 \(\mu\)M, this negative effect emerges at 0.35 \(\mu\)M Ca\(^{2+}\). The dynamics of NFAT\(_{\text{nuc}}\) and MCIP in WT under different Ca\(^{2+}\) stimulations are shown in Fig. 6 where the maximum values of NFAT\(_{\text{nuc}}\) and MCIP increase and their rising time to reach the maximum values gets shortened as Ca\(^{2+}\) increases.

4. Discussion

In this paper, we have proposed a hypothesis explaining the role change of MCIP realized by a switching feedback mechanism in the CaN-NFAT signaling pathway. The development of the mathematical model and the computer simulations have revealed the dynamics of CaN-NFAT signaling pathway in a quantitative way. We have also found that the initial concentration of the CaN/MCIP complex is important in determining the functional role of MCIP. Sanna et al. [6] did not agree with the inhibitory effect of MCIP reported by the over-expression approach. Instead, they explained the inhibition as a sequestration effect meaning that if kinase or phosphatase docking/activating proteins are over-expressed then they can intrinsically have an inhibitory function. Contrary to this, we proposed a hypothesis from a different perspective, supported by experimental evidence and quantitative analysis through mathematical modeling and simulations.

Let us consider the following simple example to further investigate the dynamics of a feedback loop system with a switching mechanism:

\[
\frac{dx}{dt} = S - a\cdot x - b\cdot y,
\]

\[
\frac{dy}{dt} = g\cdot x - d\cdot y \quad \text{with} \quad b = b_0 \frac{y^n}{k_m^n + y^n} \quad \text{or} \quad b = b_0
\]

where \(b_0\) denotes a variable gain with the threshold of \(k_m\), \(b_0\) is a constant gain, \(S\) is an input signal, and \(y\) is an output signal. Here, the switching feedback mechanism is realized by the variable gain \(b_0\). If the input signal increases the output signal above the threshold \(k_m\), the variable gain \(b_0\) converges to the constant gain \(b_0\); however, if the output signal remains below \(k_m\), the variable feedback gain gets significantly attenuated below \(b_0\). Fig. 7A and C and B and D shows the dynamics of the negative feedback loop system with \(b = b_0\frac{y^n}{k_m^n + y^n}\) and that with \(b = b_0\), respectively. For a relatively high input signal (\(S = 0.9\)), the maximal output with the variable gain is about 35% larger than that with the constant gain; however, for a relatively low input signal (\(S = 0.1\)), the maximal output with the variable gain is about 130% larger than with the constant gain. Therefore, the switching feedback mechanism plays an important role in the cut-off effect of a negative feedback loop system for a low input signal. In intracellular signaling systems, the switching feedback mechanism may serve to keep homeostasis or a constant equilibrium of the cellular system from excessive external stimuli and to adapt to other allowable stimuli.

The present paper described the functional role of MCIP in the regulation of the CaN-NFAT signaling pathway. The presented results may provide fundamental information to the development of anti-hypertrophic drug agents interfering with the CaN-NFAT signaling pathway or immuno-suppressive drug agents related to MCIP.

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