Published in final edited form as:
J Mol Cell Cardiol. 2010 February ; 48(2): 387. doi:10.1016/j.yjmcc.2009.10.023.

The mAKAPβ Scaffold Regulates Cardiac Myocyte Hypertrophy via Recruitment of Activated Calcineurin

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Abstract

mAKAPβ is the scaffold for a multimolecular signaling complex in cardiac myocytes that is required for the induction of neonatal myocyte hypertrophy. We now show that the pro-hypertrophic phosphatase calcineurin binds directly to a single site on mAKAPβ that does not conform to any of the previously reported consensus binding sites. Calcineurin - mAKAPβ complex formation is increased in the presence of Ca^{2+}/calmodulin and in norepinephrine-stimulated primary cardiac myocytes. This binding is of functional significance because myocytes exhibit diminished norepinephrine-stimulated hypertrophy when expressing a mAKAPβ mutant incapable of binding calcineurin. In addition to calcineurin, the transcription factor NFATc3 also associates with the mAKAPβ scaffold in myocytes. Calcineurin bound to mAKAPβ can dephosphorylate NFATc3 in myocytes, and expression of mAKAPβ is required for NFAT transcriptional activity. Taken together, our results reveal the importance of regulated calcineurin binding to mAKAPβ for the induction of cardiac myocyte hypertrophy. Furthermore, these data illustrate how scaffold proteins organizing localized signaling complexes provide the molecular architecture for signal transduction networks regulating key cellular processes.

Keywords

calcineurin; mAKAP; NFATc; hypertrophy; protein complex; signaling

1. Introduction

Cardiac myocyte hypertrophy is the major intrinsic mechanism by which the heart may counterbalance chronically elevated demands for pumping power. Myocyte hypertrophy is controlled by a network of intracellular signaling pathways that are activated by G-protein coupled, growth factor and cytokine receptors and by mechanical and oxidative stress [1]. These signals are transduced by MAPK, cyclic nucleotide, Ca^{2+} and phosphoinositide-dependent pathways. Although much progress has been made over the last twenty years to

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define this network, it is still unclear how the various constituent pathways act in concert to regulate the overall cellular phenotype [2]. Moreover, while individual signaling pathways may regulate specific cellular functions, the molecules that comprise these signaling pathways often serve multiple functions in the same cells. Therefore, an important question in the field of signal transduction has been how pleiotropic signaling molecules such as protein kinases and phosphatases can specifically regulate individual downstream effectors in response to different upstream stimuli. One mechanism by which specificity in signal transduction is conferred is the formation of multimolecular signaling complexes by scaffold proteins of different combinations of common signaling enzymes [3].

While signaling enzymes may be broadly distributed within the cell, scaffold proteins, such as A-kinase anchoring proteins (AKAPs), recruit small pools of these enzymes to discrete multimolecular complexes that are sequestered in distinct intracellular compartments and that serve different cellular functions [4]. mAKAP (muscle AKAP) was initially identified in a screen for protein kinase A (PKA) binding proteins. mAKAPα and mAKAPβ are the two known isoforms encoded by the single *mAKAP (AKAP6)* gene and are expressed in neurons and striated myocytes, respectively [5]. As a consequence of alternative mRNA splicing, mAKAPβ is identical to residues 245 – 2314 (the C-terminus) of mAKAPα. In adult and neonatal cardiac myocytes, mAKAPβ is primarily localized to the outer nuclear membrane through its association with nesprin-1α [6,7]. In addition to PKA, proteins that have been shown to associate with the mAKAPβ scaffold in myocytes include adenylyl cyclase type 5 [8], the cAMP-specific phosphodiesterase PDE4D3 [9], the cAMP-activated guanine nucleotide exchange factor Epac1 [10], ERK5 and MEK5 mitogen-activated protein kinases (MAPK) [10], the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin Aβ (CaN, PP2B) [11], protein phosphatase 2A [12], hypoxia-inducible factor 1α (HIF1α) and ubiquitin E3-ligases involved in HIF1α regulation [13], myopodin [14], the ryanodine receptor Ca^{2+}-release channel (RyR2) [12,15] and the sodium/calcium exchanger NCX1 [16]. Due to the association of these various enzymes and ion channels with mAKAPβ in the cardiac myocyte, we have proposed that mAKAPβ complexes are important for the regulation of pathologic myocyte remodeling in response to upstream cAMP, calcium, and MAPK signals and hypoxic stress [13,17]. In support of this hypothesis, mAKAPβ expression in myocytes is required for the full induction of neonatal myocyte hypertrophy in vitro by adrenergic and cytokine agonists [10,11].

CaN is a pleiotropic Ca^{2+}/calmodulin-dependent serine/threonine phosphatase composed of a catalytic A-subunit and a regulatory B-subunit [18]. There are three mammalian A-subunits, of which Aα and Aβ are expressed ubiquitously and Aγ is restricted to testes. Aα and Aβ have been studied by genetic deletion and are not functionally redundant. For example, only the CaNAβ isoform is important for the induction of pathologic cardiac hypertrophy and the survival of myocytes after ischemia [19,20]. Important calcineurin substrates in vivo include four of the five members of the nuclear factor of activated T-cell transcription factor family (NFATc 1–4). In addition to forming heterodimers with other transcription factors, NFATc can bind directly to CaN through conserved PxIxIT and LxVP motifs [21]. CaN binding facilitates dephosphorylation of the N-terminal NFATc regulatory domain, inducing NFATc nuclear translocation from the cytoplasm. Accordingly, NFATc isoforms serve important roles in cardiac development and myocyte hypertrophy [22].

Previously, we showed that CaNAβ is associated with mAKAPβ in cardiac myocytes [11]. However, it remains unclear how scaffolding by this relatively low abundant protein contributes to CaN signaling. In this study, we characterize the direct binding of CaNAβ to mAKAPβ. Moreover, we provide evidence that recruitment of CaNAβ and NFATc3 to mAKAPβ complexes is important for the transduction of hypertrophic signaling.

2. Materials and Methods

2.1 Antibodies and antiserum

Commercially available antibodies were as follows: rabbit and mouse anti-Flag (Sigma), anti-S tag (Novagen), anti-His tag (Santa Cruz), mouse anti-HA tag (Sigma), mouse anti-myc tag (monoclonal 4A6, Millipore), rabbit anti-CaNAβ (Santa Cruz), mouse anti-NFATc1 (BD Biosciences), rabbit anti-NFATc3 (Santa Cruz), rabbit anti-CaNAβ (Santa Cruz), mouse anti-α-actinin (monoclonal EA-53, Sigma), rabbit anti-rat atrial natriuretic factor (ANF; US Biological), horseradish peroxidase (HRP)-conjugated donkey secondary antibodies (Jackson ImmunoResearch) and Alexa dye-conjugated donkey secondary antibodies (Molecular Probes). HRP-conjugated VO145, OR010 (Covance), VO56, and VO54 rabbit and 720 (Covance) mouse anti-mAKAP antibodies were as previously described [5–7].

2.2 Expression Vectors

The rat mAKAP siRNA and control siRNA expression plasmids and the mammalian expression plasmids (pCDNA3.1 (-) mychis vector, Invitrogen) for wildtype rat mAKAPα and mAKAPβ are as previously described [11,12]. Full-length deleted forms of rat mAKAPβ were generated by site-directed mutagenesis using the Quickchange method (Stratagene) and sense and antisense oligonucleotides to the following sequences: del 1301–1400 GAGGACAGCCCACTGGGATGCAGCCAATG; del 1401–1500 CCGGACCCCAAATGTATTTTGTAAAAAGTCCTGC; del 1501–1600 - CCCCTTCTTGGTGGTTTTTATAAGACAATGAGGATCTC. A Flag-tagged full-length NFATc3 expression vector was constructed using a cDNA provided by Dr. Neil Clip-stone. HA-tagged CaNAβ expression vectors were constructed using a cDNA obtained by PCR using mouse brain cDNA. Adenovirus that express the various proteins were generated using the Adeno-X Tet-Off System (Clontech) [12]. All bacterial expression vectors were constructed by subcloning relevant PCR products into the pET30 (Novagen) or pGEX4T parent vectors (Pharmacia). All plasmid constructs were verified by sequencing, and details of the various constructions are available upon request. pET15 bacterial expression vectors for PKA catalytic subunit and GSK-3β were the gifts of Dr. Susan Taylor and Dr. Peter Roach, respectively. pET15-CaNAβB and pBB131 vectors were the gift of Jun O. Liu [23].

2.3 Ventricular Myocyte Culture

Ventricular myocytes (over 90% free of fibroblasts) were prepared from 2–3-day old Sprague-Dawley rats, as previously described [6]. The cells were plated in Dulbecco's Modified Eagle medium (DMEM) with 17% Media 199, 1% penicillin/streptomycin solution (P/S, Gibco-BRL), 10% horse serum and 5% fetal bovine serum (FBS) at 32,000 and 125,000 per cm^2 for immunocytochemical and biochemical experiments, respectively. After overnight in plating medium, the myocytes were maintained in culture for up to one week in maintenance medium (79% DMEM, 20% Media 199, and 1% P/S) supplemented with 50 μM phenylephrine before use. For adenoviral-based expression, the myocytes were infected with adenovirus (MOI = 15–100) using the Adeno-X Tet-Off System (Clontech). For plasmid-based expression, the myocytes were transfected with Transfast (Promega) as suggested by the manufacturer. Transfection efficiencies were typically between 1–5%.

Myocyte immunocytochemistry and morphometrics was performed as previously described by digital wide-field fluorescent microscopy using IPLab 4.0 software (BD Biosciences) [11]. All data are expressed as mean ± s.e.m. Each n represents the results of experiments using separate primary cultures. Within each experiment, ~25 cells were measured for each condition for both morphometric and ANF expression studies. ANOVA was calculated as a single factor with $\alpha = 0.05$; individual p-values were calculated using two-tailed, paired Student's T-tests.

For NFAT reporter assays, neonatal myocytes (120,000/cm^2) were transfected with Transfast and cultured for 48 hours before analysis using the Dual Luciferase Reporter Assay System (Promega) and a Berthold Centro X luminometer. The NFAT- firefly luciferase reporter vector containing nine NFAT binding sites 5′ to the -164 αMHC minimal promoter was a gift of Jeffrey Molkentin [24]. The -164 αMHC minimal promoter was inserted into pRL-null (Promega) to provide the control renilla luciferase vector.

2.4 Other Cell Culture

HEK293 and COS-7 cells were maintained in DMEM with 10% FBS and 1% P/S. These cells were transiently transfected with Lipofectamine 2000 (Invitrogen) or infected with adenovirus and Adeno-X Tet-Off virus (Clontech) as suggested by the manufacturers.

2.5 Co-Immunoprecipitation

For immunoprecipitation, tissue were homogenized using a Polytron or cells were lysed in IP buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton-X 100, 1 mM DTT) plus an inhibitor cocktail (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM benzamidine, 1 mM AEBSF, 50 mM NaF, 1 mM sodium orthovanadate). Soluble proteins were separated by centrifugation at 20,000 *g* for 10 minutes. Antibody and 10 μl pre-washed protein-A or protein-G agarose beads (50% slurry, Upstate) or 10 μl HA or Flag antibody-conjugated sepharose beads (Sigma) were added to extracts and incubated overnight with rocking at 4°C. Beads were washed three times for 5 minutes at 4°C with IP buffer. Bound proteins were size-fractionated on SDS-PAGE gels and developed by immunoblotting as previously described using either X-ray film or a Fujifilm LAS-3000 imaging system [6]. Protein markers were Precision Plus Protein Standards (Bio-Rad).

2.6 Pull-down assays

GST and His-tag fusion proteins were purified from the soluble bacterial fraction using His-bind (Novagen) and Glutathione Uniflow Resins (Clontech). Active, myristoylated CaNAβ holoenzyme was purified as previously described using both His-bind resin and calmodulin-sepharose affinity chromatography (Sigma) [23]. Purified His-tagged proteins were mixed in buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM DTT and protease inhibitors) and pulled down using Glutathione Uniflow Resin previously adsorbed with GST fusion protein. Beads were washed with buffer containing 1% Triton X-100 and proteins analyzed as above. Proteins expressed using pET30 expression plasmids include both His_6 and S tags N-terminal to the protein of interest and were detected with either anti-His or anti-S-tag conjugated antibodies.

2.7 CaN Phosphatase Assay

CaN substrate was prepared as follows: 6 μg bacterially-expressed GST-NFATc1 2–418 fusion protein adsorbed to glutathione-uniflow resin (Clontech) was incubated for 1 hour at 37°C in kinase buffer (20 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT) with purified bacterially-expressed His-tagged GSK-3β and PKA catalytic subunit (1 μg each), 100 pmol [^{32}P-γ]-ATP (7000 Ci/mmol, 23 μM) and 0.1 mM cold ATP. The reaction was stopped by washing the beads 12 times with PBS containing 5 mM EDTA and then with PBS (no EDTA) until counts in the wash were about 100x less that the counts remaining on the beads. The beads were resuspended in five volumes PBS and an aliquot was measured by liquid scintillography such that the specific activity was greater than 5 μCi/μg protein.

Bacterially-expressed GST-mAKAP 1240–1346 or GST alone (35 μg each) adsorbed to glutathione-uniflow resin (Clontech) were incubated overnight at 4°C in phosphatase buffer (50mM Tris pH 7.4, 100mM NaCl, 8 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 mM DTT) with 13 nM

bacterially purified, His-tagged CaNAβ holoenzyme, 0.5% BSA, and 1 μM calmodulin (bovine testes, Sigma) in the presence or absence of 25 mM EGTA. Samples were washed 4 times for 3 minutes with phosphatase buffer, and the beads were resuspended in 500 μl phosphatase buffer with 0.5% BSA, 1 μM calmodulin. 100 μl aliquots of beads and buffer were used for phosphatase assay in the presence or absence of 25 mM EGTA. [^{32}P]GST-NFATc1 2–418 on glutathione resin was diluted with stock glutathione resin to 5 Mcpm per 20 μl bed volume in 1.8 ml phosphatase buffer. To start the reaction, 75 μl beads in buffer were added to the 100 μl aliquots of pulled-down protein and incubated for 30 minutes at 37°C in a Thermomixer (Eppendorf). The assay was stopped by adding 500 μl PBS with 5 mM EDTA. The samples were briefly micro-fuged at full speed, and the separate supernatant and resin were analyzed by liquid scintillography. Assay conditions were adjusted to ensure no more than 10% dephosphorylation of the substrate during each reaction.

3. RESULTS

3.1 Binding of mAKAPβ and CaN in myocytes

As we have shown previously [11], endogenous CaNAβ and mAKAPβ associated in adult rat heart extracts can be co-immunoprecipitated with a mAKAPβ-specific antibody (Fig. 1B). In order to test whether mAKAPβ can also bind CaNAα, HEK293 cells were transfected with expression vectors for mAKAPβ and either CaNAα or CaNAβ HA-tagged isoforms (Fig. 1C). mAKAPβ was efficiently co-immunoprecipitated when either HA-tagged CaN isoform was present (lanes 2 and 3). In addition, mAKAPβ was co-immunoprecipitated with a constitutively active mutant of CaNAβ containing residues 1–412 (Ha-CaNAβca, Fig 1D), lacking the CaNA autoinhibitory domain (AID, Fig. 1A).

We have shown that mAKAPβ expression is required for the adrenergic-induced hypertrophy of cultured neonatal rat myocytes [11]. Therefore, we were interested whether mAKAPβ-CaNAβ binding was regulated in these cells. Norepinephrine (NE) stimulation of adrenergic receptors resulted in a slow, but significant increase in mA-KAPβ-CaNAβ association as detected by co-immunoprecipitation assay of myocytes expressed HA-tagged CaNAβ (Fig. 1E, lane 6).

3.2 A discrete domain of mAKAPβ binds CaNAβ in a Ca^{2+}/calmodulin-dependent manner

In order to map the binding domain on mAKAP required for CaN association, we used bacteria to express S-tagged CaNAβ subunit and glutathione-S-transferase (GST) mAKAP fusion proteins (Fig. 2B). These proteins are shown in Fig. 2A in alignment to the binding sites that have been mapped for other mAKAPβ protein partners. Purified GST-mAKAP fusion proteins containing residues 1286–1833 consistently pulled down Stagged-CaNAβ (Fig. 2B, lane 4). Fragments containing other mAKAP sequences, including the mAKAPα-specific N-terminal domain (lane 2), were unable to bind CaNAβ. Further mapping revealed that GST-mAKAPβ 1240–1346 could pull down His-tagged CaNAβ (Fig. 2C). Conversely, GST-CaNAβ fusion protein, but not GST alone, was able to pull-down a His-tagged mAKAP fragment containing residues 1286–1345 (Fig. 2D, lane 3).

The requirement of this CaN binding site was tested using a series of internally deleted, full-length mAKAPβ mutant proteins (Fig. 2E and Sup. Fig. 1). HA-tagged CaNAβ was co-expressed in COS-7 cells with myc-tagged wildtype or mutant mAKAPβ proteins and analyzed by co-immunoprecipitation assay. While wildtype mAKAPβ was effectively co-immunoprecipitated with HA-CaNAβ (Fig. 2E), an mAKAPβ mutant lacking residues 1301–1400 poorly associated with CaNAβ in cells (lane 4). Similar results were obtained in co-immunoprecipitation experiments using HA-CaNAβca protein (Sup. Fig. 1). Together, these

data are consistent with the results of the mapping using bacterially-expressed proteins (Fig. 2B–D) and with a predominant site on mAKAPβ for CaNAβ binding.

Several CaN consensus binding sequences have been reported in the literature [21]. Of note, primary sequence analysis of mAKAP residues 1286–1345 failed to demonstrate any known CaN binding consensus sites, nor any significant homology to other known proteins. These results suggest that mAKAP 1286–1345 contains a novel CaN binding site.

3.3 Regulation of mAKAP-CaN Binding

Because CaNAβ-mAKAPβ binding was induced by NE in myocytes, we tested whether CaNAβ-mAKAPβ binding was regulated by Ca^{2+}/calmodulin-dependent activation in vitro. GST-pull down assays were performed using purified GST-mAKAP 1240–1346, His-tagged CaNAβ holoenzyme, and calmodulin in the presence or absence of Ca^{2+} (Fig. 2C). His-CaNAβ bound GST-mAKAP 1240–1346 (lanes 2 and 4), but not GST alone (lanes 1 and 3). Importantly, the protein-protein interaction was significantly increased in the presence of Ca^{2+} (lane 2 vs. 4).

Given that CaNAβ bound more strongly to mAKAP 1240–1346 in the presence of Ca^{2+}/calmodulin, we suspected that the mAKAP-bound CaN was catalytically active. GST-mAKAP 1240–1346 pull-downs were repeated in the presence of Ca^{2+}/calmodulin, and the precipitated protein was analyzed by CaN phosphatase assay using PKA- and GSK-3β-phosphorylated NFATc1 regulatory domain (residues 2–418) as substrate (Fig. 3). CaNAβ bound to mAKAP was able to dephosphorylate the non-peptide, protein substrate in a Ca^{2+}-dependent manner. Important controls were that CaN activity was weakly detected in control pull-downs with GST alone (left bars) or after pull-down with GST-mAKAP 1240–1346 either in the absence of Ca^{2+} or in the presence of a competing His-mAKAP 1286–1345 peptide (Sup. Fig. 2).

3.4 Functional Significance of CaN-mAKAP Binding

Previously, we discovered using RNA interference (RNAi) that mAKAPβ expression is required for the full induction of neonatal myocyte hypertrophy in vitro by the α- and β-adrenergic agonists phenylephrine and isoproterenol, as well as by leukemia inhibitory factor [10,11]. We now show in Fig. 4 that CaNAβ binding to mAKAPβ is required for neonatal myocyte hypertrophy by comparing myocytes expressing myc-tagged wildtype mAKAPβ (myc-mAKAPβ WT) or mAKAPβ Δ1301–1400 (myc-Δ1301–1400) which does not bind CaNAβ (Fig. 2E).

In order to express recombinant mAKAPβ without any background expression of endogenous mAKAPβ, we utilized mAKAPβ siRNA to deplete the native protein. Myocytes were co-transfected with plasmids expressing a mAKAP-specific small interfering RNA (siRNA) and green fluorescent protein (GFP) (Sup. Fig. 3, panels e-h). GFP-positive myocytes showed diminished mAKAPβ staining by immunocytochemistry when compared to non-transfected cells or myocytes expressing a control siRNA (Sup. Fig. 3, panels a–d). Subsequently, mAKAPβ RNAi was rescued by co-transfection of a third plasmid expressing myc-mAKAPβ WT (Fig. 4A and B, panels a–d) or myc-Δ1301–1400 (Fig. 4A and B, panels e–h). Importantly, we only studied GFP-positive myocytes expressing recombinant, myc-tagged mAKAPβ at approximately the same abundance and localization as endogenous mAKAPβ in adjacent, non-transfected cells (Fig. 4A, panels c and g).

Myocyte hypertrophy was assayed by measuring myocyte cross-section area on GFP images. In these experiments, NE-treatment of myocytes expressing myc-mAKAPβ WT increased myocyte cross-section area 24 ± 8 % (Fig. 4C). Significantly, NE-treated myocytes expressing myc-Δ1301–1400 (Fig. 4A, panels e–h) were smaller in cross-section area than those

expressing myc-mAKAPβ WT (11 ± 8 % increased cross-section area compared to control myc-mAKAPβ WT-expressing myocytes cultured in the absence of agonist). In contrast, untreated myocytes expressing myc-Δ1301–1400 were not significantly different in size (0.97 ± 0.06 fold cross section area) from untreated myocytes expressing myc-mAKAPβ WT. Staining for ANF expression constituted a second, independent assay for hypertrophy (Fig. 4B). Adrenergic stimulation induced ANF expression in 36 ± 4 % of myocytes expressing myc-mAKAPβ WT in contrast to only 23 ± 6 % of myocytes expressing the mutant protein (Fig. 4D). Together, these findings are consistent with a requirement for CaN binding to the mAKAPβ scaffold in NE-induced hypertrophic signaling.

3.5 NFATc3 and mAKAPβ Associate in the Heart

Having shown that CaN binding to mAKAPβ was relevant to hypertrophic signaling in vitro, we were interested in identifying potential substrates for the phosphatase that might also dock on the scaffold protein. We tested whether mAKAPβ might bind NFATc3 transcription factor by immunoprecipitating complexes from adult rat heart extracts with mAKAP-specific antiserum (Fig. 5A). NFATc3 was reproducibly detected in mAKAP-specific immunoprecipitates (Fig. 5A, lane 3), but not in control, preimmune immunoprecipitates (Fig. 5A, lane 2). In order to confirm the association of NFATc3 with mAKAPβ, we infected primary myocytes with adenovirus that express flag-tagged NFATc3 and myc-tagged mAKAPβ (Fig. 5B). mAKAPβ was immunoprecipitated with anti-Flag antibody only when co-expressed with Flag-tagged NFATc3 (Fig. 5B, lane 3).

We predicted that NFATc3 associated with mAKAPβ would be dephosphorylated by CaNAβ bound to the scaffold. When complex formation was driven by overexpression of both mAKAPβ and NFATc3 in myocytes, we found that Flag-tagged NFATc3 migrated faster on SDS-PAGE, consistent with a decreased phosphorylation state (Fig. 5B lane 3, and Fig. 5C lane 2). Remarkably, CaNAβ binding to mAKAPβ was required for NFATc3 dephosphorylation as overexpression of the mAKAPβ mutant lacking CaNAβ binding (myc-Δ1301–1400) had no effect on NFATc3 phosphorylation state (Fig. 5C, lane 3).

Next we tested whether mAKAPβ was required for NFAT transcriptional activity in myocytes by transient luciferase reporter assay (Fig. 5D). As expected, α-adrenergic stimulation resulted in a 2-fold increase in specific NFAT activity, which was completely inhibited by addition of the CaN inhibitor cyclosporine A (CsA). Importantly, inhibition of mAKAPβ expression by RNAi also inhibited phenylephrine-induced NFAT activity, while not affecting either baseline or CsA-inhibited reporter activity. As a control, mAKAPβ RNAi was reversed by rescue using the myc-mAKAPβ WT expression vector, restoring α-adrenergic-induced NFAT activity. Taken together, these data show that expression of the mAKAPβ scaffold is required for NFAT transcriptional activity in cultured myocytes.

4. DISCUSSION

In this paper we present evidence that CaNAβ forms inducible complexes with the scaffold protein mAKAPβ that are important for the transduction of hypertrophic signaling. In contrast to CaN scaffolds that have been previously described such as calsarcin, AKAP79/150 and AKAP121 which inhibit CaN activity and may compete for NFATc binding [25–27], CaNAβ bound to a mAKAPβ fusion protein actively dephospho-rylated phospho-NFATc3 substrate (Fig. 5C). Moreover, CaNAβ-mAKAPβ binding was enhanced in vitro by Ca^{2+}/calmodulin (Fig. 2C).

It is well established that CaNAβ and NFATc transcription factors contribute to the induction of cardiac myocyte hypertrophy [28]. Using primary myocyte cultures expressing a properly localized mAKAPβ mutant lacking the CaN-binding domain, we were able to detect a specific

requirement of CaN binding to mAKAPβ for NE-induced hypertrophy (Fig. 4), consistent with our observation that NE induced CaNAβ-mAKAPβ binding in cells (Fig. 1E). In the myocyte, CaN and NFATc are enriched at the Z-disk, where CaN may be inhibited by calsarcin-1 [29]. This sarcomeric structure is considered an important center for the activation of hypertrophic signaling, including for activation of NFATc transcription factors [30–32]. In other cellular compartments, CaN may be inhibited by other scaffolds such as AKAP121, whose knock-down results in enhanced myocyte hypertrophy [27]. Upon stimulation, there is evidence that both CaN and NFATc shuttle to the nucleus to promote hypertrophy [33]. Our data support a model in which activated CaNAβ translocates towards the nucleus, where a small pool of CaNAβ may be recruited to mAKAPβ signaling complexes.

At the mAKAPβ "signalosome," we propose that CaNAβ promotes the activation of NFATc transcription factors that can contribute to myocyte hypertrophy. By co-immunoprecipitation assays (Fig. 5), we found that NFATc3 and mAKAPβ associate in cardiac myocytes, whereby NFATc3 might be dephosphorylated by mAKAPβ-bound CaNAβ (Fig. 5C). Like CaNAβ, NFATc3 has been shown to be required in vivo for the induction of pathologic hypertrophy [19,34]. In preliminary experiments, mAKAPβ does not appear to bind NFATc3 either directly or through a discrete domain (data not shown). Nevertheless, the association of NFATc3 and CaNAβ with mAKAPβ appears to be functionally significant, since mAKAPβ expression was required for adrenergic-induced NFAT transcriptional activity (Fig. 5D). mAKAPβ may serve a more general role in the regulation of NFATc transcription factors in striated muscle, as we have previously shown that mAKAPβ expression was required for adrenergic-induced NFATc1 nuclear translocation [11], and because NFATc1 and NFATc4 can also be co-immunoprecipitated with mAKAPβ from myocyte extracts (data not shown).

In conclusion, we present the novel findings that CaNAβ recruitment to the mAKAPβ signalosome is important for NE-induced myocyte hypertrophy in vitro. These data underscore the importance of CaN anchoring and localization to adrenergic signaling. Because of its role in hypertrophic signaling, the mAKAPβ signalosome may represent a novel target in the prevention or treatment of pathologic hypertrophy and heart failure. Because CaN binds a non-consensus site on mAKAPβ, inhibition of CaNAβ-mAKAPβ complex formation may represent an alternative strategy to the global inhibition of CaN activity or to the inhibition of CaN binding to the many scaffold proteins and substrates that contain PxIxIT sites. Since mAKAPβ is a relatively low abundant protein enriched at the nuclear envelope [12], mAKAPβ may only bind a small fraction of total cellular CaNAβ and NFATc transcription factors at any given moment. Thus, these data exemplify how individual, discretely localized signaling complexes can control key cellular processes by dynamically integrating multiple upstream signals. The pharmacologic targeting of such downstream effector complexes may provide the specificity that is lacking in many current therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by National Heart, Lung, and Blood Institute grant RO1 HL075398 to M.S.K. and HL082705 to K.D.K.

Abbreviations

AID	autoinhibitory domain
ANF	atrial natriuretic factor

CaN	calcineurin
CaNAβca	constitutively active mutant of CaNAβ
CsA	cyclosporine A
GFP	green fluorescent protein
GST	glutathione-S-transferase
mAKAPβ	muscle A-kinase anchoring protein
NE	norepinephrine
NFATc	nuclear factor of activated T-cell
PE	phenylephrine
PKA	protein kinase A
RyR2	ryanodine receptor

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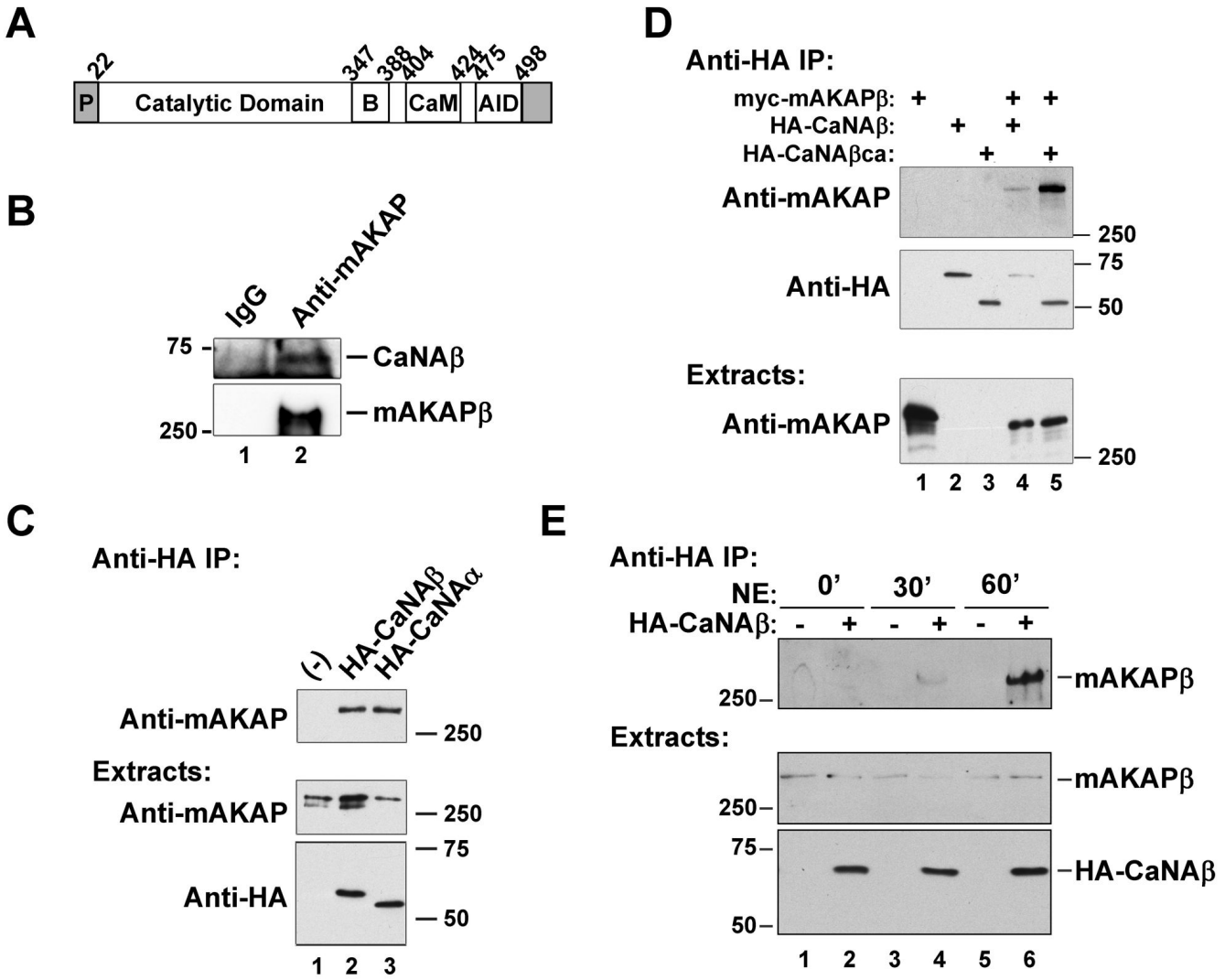

Fig. 1.

CaN binds mAKAPβ in cardiac myocytes. *A.* Structure of CaNAβ [35]. Conserved CaNA domains include the catalytic domain, the B-subunit (B) and calmodulin (CaM) binding domains, and the autoinhibitory domain (AID). The non-conserved N-terminal polyproline and C-terminal domains are in gray. *B.* Protein complexes were immunoprecipitated from adult rat heart extracts with α-mAKAP monoclonal 720 antibody (lane 2) or control mouse IgG (lane 1). mAKAPβ and CaNAβ were detected by western blotting with specific rabbit antibodies. *C.* Protein complexes were immunoprecipitated with HA-conjugated sepharose beads from extracts prepared from HEK293 cells expressing myc-mAKAPβ and/or HA-tagged CaNAβ and CaNAα. Total extracts (bottom panels) and immunoprecipitates (top panel) were probed for the presence of mAKAP and CaNA using HRP-conjugated mAKAP antibody and a HA antibody, respectively. *D.* Protein complexes prepared from COS-7 cells expressing myc-mAKAPβ and/or HA-tagged CaNAβ wildtype or constitutively active mutant (residues 1–412, HA-CaNAβca) were isolated and analyzed as in C. *E.* Primary myocytes expressing HA-tagged CaNAβ were stimulated with 10 μM NE for the indicated times. Cell lysates were immunoprecipitated with a monoclonal HA-antibody. Endogenous mAKAPβ and HA-

CaNAβ were detected with VO54 mAKAP and HA antibodies, respectively. For all panels, n 3, and molecular mass markers are indicated in kDa.

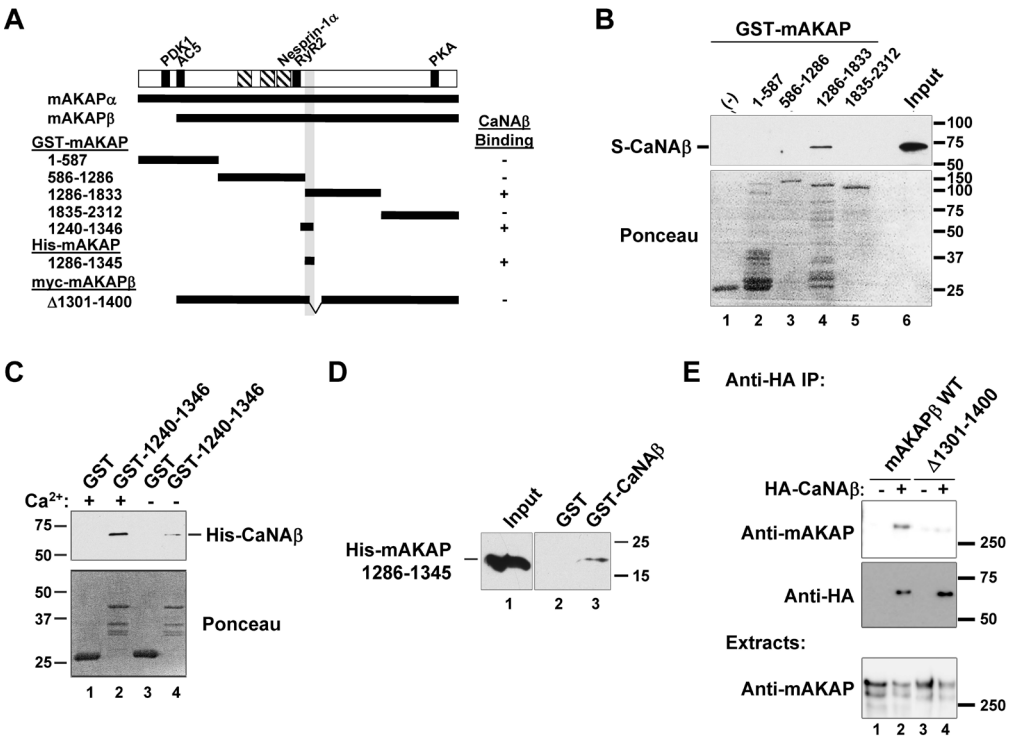

Fig. 2.

CaNAβ binds a discrete mAKAPβ domain in a Ca^{2+}/calmodulin-dependent manner. *A.* Diagram showing mAKAP domains and fragments used in these experiments. mAKAPβ is equivalent to mAKAPα 245–2314. Binding sites are indicated for PDK1 (residues 227–232 [5]), adenylyl cyclase 5 (AC5, 275–340 [8]), nesprin-1α (1074–1187 [7]) RyR2 (1217–1242 [36]), and PKA (2055–2072 [6]). The spectrin-like repeat domains (green, residues 772–1187 [6]) are hatched. A gray bar highlights the mapped CaN-binding domain. *B.* 1 μg purified His- and S-tagged CaNAβ subunit (30 nM, 2% input shown in lane 6) was subjected to pull-down assay by ~10 μg GST-mAKAPβ fusion proteins (lanes 2–5) or GST alone (lane 1) and was detected by anti-S-tag antibody (top panel). GST fusion proteins present in the assay were detected by Ponceau S total protein stain of the same blots (bottom panel). *C.* Purified His-tagged CaNAβ holoenzyme was used in GST-pull-down assays as in B, except that the buffer contained 1.5 mM $CaCl_2$ and 1 μM calmodulin. EDTA was added to chelate Ca^{2+} as indicated (lanes 3 and 4). Associated His-CaNAβ was detected using CaNAβ antibody (top panel). *D.* 10 μg His-mAKAP 1286–1345 (1.5 μM, lane 1) was subjected to GST pull-down assay in buffer containing Ca^{2+}/calmodulin using 0.5 μg GST-CaNAβ (lane 3) or GST alone (lane 2) and detected by anti-His antibody. *E.* Protein complexes were immunoprecipitated with HA antibody from extracts prepared from HEK293 cells expressing HA-tagged CaNAβ and myc-mAKAPβ wildtype or myc-mAKAP Δ1301–1400 (Δ1301–1400). Total extract (bottom panels) and immunoprecipitates (top panel) were probed for the presence of mAKAPβ and CaNAβ using mAKAP and HA antibodies, respectively. n 3 for each panel.

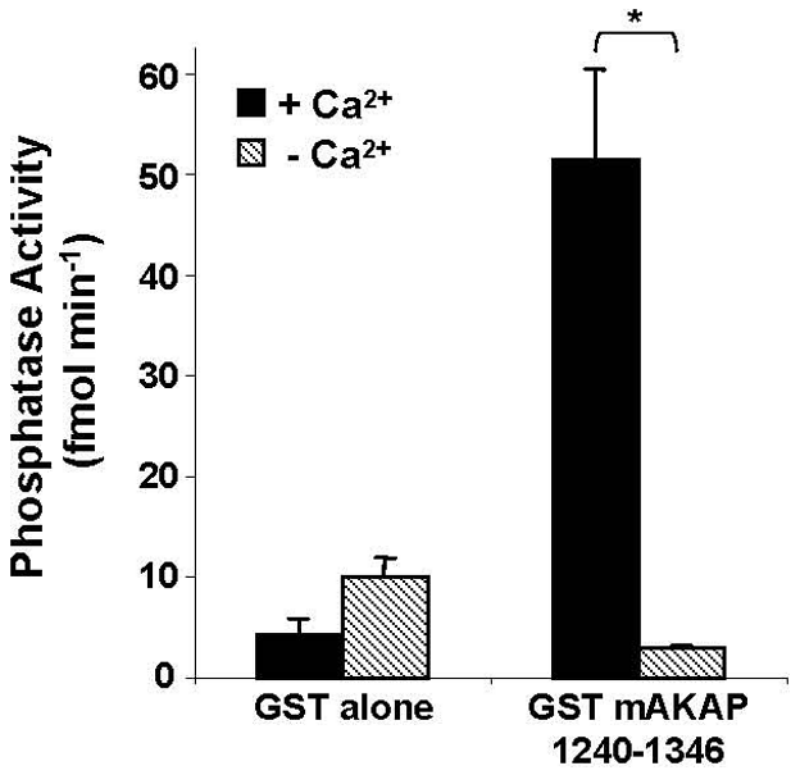

Fig. 3.

CaNAβ bound to mAKAP is catalytically active. Purified CaNAβ holoenzyme (13 nM) was subjected to pull-down assay by GST-mAKAP 1240–1346 or GST alone in the presence of Ca^{2+}/calmodulin. Activity in the precipitates was assayed using ^{32}P-NFATc1 substrate in the presence and absence of Ca^{2+}. *$p < 0.05$; n=3.

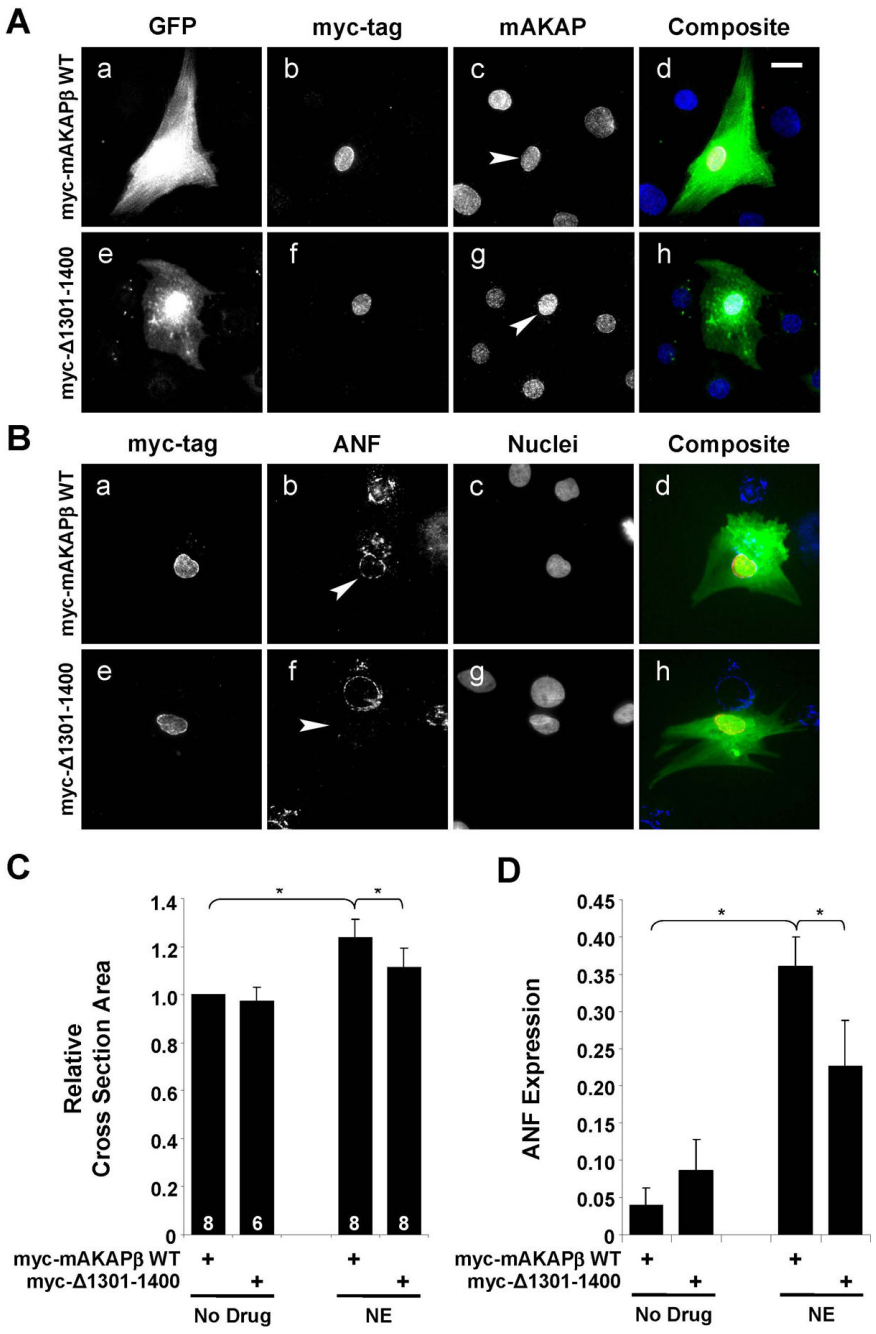

Fig. 4.

CaN-mAKAPβ binding contributes to norepinephrine-stimulated myocyte hypertrophy in vitro. *A and B.* Primary rat neonatal ventricular myocytes were co-transfected with expression vectors for GFP, mAKAP siRNA, and either myc-tagged mAKAPβ WT (panels a–d) or mAKAPβ Δ1301–1400 (panels e–h) and cultured for two days ± 10 μM NE. Representative wide-field images of NE-treated myocytes are shown. Note that in wide-field images, as opposed to confocal images, nuclear envelope and intra-nuclear staining are indistinguishable [7]. Cells were stained with myc-tag (panels b and f) and mAKAPβ-specific (panels c and g) antibodies [6]. Only GFP-expressing (panels a and e), myc-tag positive cells showing an intensity of staining with the mAKAP antibody (arrowheads, panels c and g) similar to adjacent,

non-transfected cells were studied. Panels d and h are composite images showing GFP (green), myc-tag (red) and mAKAP (blue) staining. Scale bar in panel h indicates 20 μm. *B.* Additional cells were stained with myc-tag (panels a and e) and ANF (panels b and f, arrowheads point to nuclei of transfected cells) antibodies and Hoechst DNA stain (Nuclei, panels c and g). Panels d and h are composite images showing GFP (green), myc-tag (red) and ANF (blue) staining. Only cells showing localized myc-tag staining with an intensity similar to that in *A* were included in these studies. Scale is as in *A. C.* Fold cross-section area ± s.e.m. is indicated for each condition. The data are normalized to the values for untreated, myc-mAKAPβ WT expressing cells (1142 ± 78 μm^2). *$p \leq 0.02$; p(ANOVA) = 0.03; n, as indicated on each bar, is for the number of independent experiments. For each experiment, ~25 myocytes were studied for each condition, totaling 150–200 myocytes. *D.* The fraction of cells stained with the ANF antibody ± s.e.m. is indicated for each condition. *$p < 0.04$; p(ANOVA) = 0.003; n = 3 independent experiments, totaling 75 myocytes per condition.

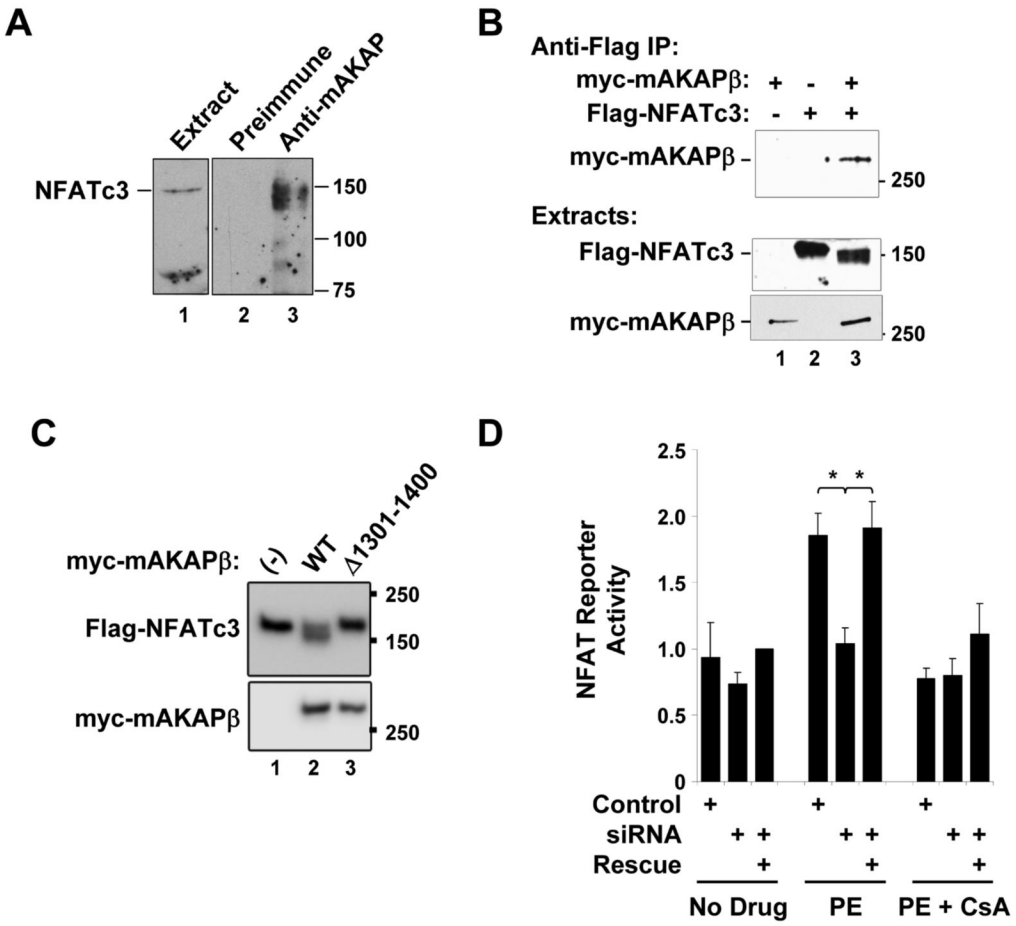

Fig. 5.

NFATc3 is a substrate for mAKAPβ-bound CaNAβ. *A.* Rat heart extract was immunoprecipitated with preimmune (lane 2) or anti-mAKAP VO54 (lane 3) antibodies. Immunoprecipitates were analyzed by Western blot using NFATc3 antibody. *B.* Primary myocytes were infected with adenoviruses expressing myc-tagged mAKAPβ and Flag-tagged NFATc3 alone (lanes 1 and 2) or in combination (lane 3). Two days following infection, cells were treated for 1 hour with 2 μM ionomycin. Cell lysates were immuno-precipitated with Flag-conjugated agarose beads. NFATc3 and mAKAPβ were detected using Flag and mAKAP antibodies, respectively. *C.* Extracts were prepared from primary myocytes infected with adenovirus for Flag-NFATc3 and myc-mAKAPβ WT and Δ1301–1400. The over-expressed proteins were detected with Flag and myc antibodies, respectively. $n \geq 3$ for A–C. *D.* Primary myocytes were co-transfected with a NFAT-firefly luciferase reporter, a renilla luciferase control reporter, and an expression plasmid for either a control or mAKAP-specific siRNA. An expression plasmid for myc-mAKAPβ was included in rescue experiments. The myocytes were treated for two days with PE and propanolol and/or CsA. Data (firefly luciferase/renilla luciferase) were normalized to the no drug, rescue samples. *$p<0.05$, $p(\text{ANOVA}) = 10^{-5}$, n = 3–6.