The mAKAP complex participates in the induction of cardiac myocyte hypertrophy by adrenergic receptor signaling

Genevieve C. Pare[1,*], Andrea L. Bauman[1,*], Molly McHenry[1], Jennifer J. Carlisle Michel[1], Kimberly L. Dodge-Kafka[2] and Michael S. Kapiloff[1,‡]

[1]Department of Pediatrics, Department of Cell and Developmental Biology, Heart Research Center, Oregon Health and Science University, NRC5 3181 S.W. Sam Jackson Park Road, Portland, OR 97239, USA
[2]Calhoun Center for Cardiology University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA
*These authors contributed equally to this work
‡Author for correspondence (e-mail: kapiloff@ohsu.edu)

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Summary

Maladaptive cardiac hypertrophy can progress to congestive heart failure, a leading cause of morbidity and mortality in the United States. A better understanding of the intracellular signal transduction network that controls myocyte cell growth may suggest new therapeutic directions. mAKAP is a scaffold protein that has recently been shown to coordinate signal transduction enzymes important for cytokine-induced cardiac hypertrophy. We now extend this observation and show mAKAP is important for adrenergic-mediated hypertrophy. One function of the mAKAP complex is to facilitate cAMP-dependent protein kinase A-catalyzed phosphorylation of the ryanodine receptor Ca^{2+}-release channel. Experiments utilizing inhibition of the ryanodine receptor, RNA interference of mAKAP expression and replacement of endogenous mAKAP with a mutant form that does not bind to protein kinase A demonstrate that the mAKAP complex contributes to pro-hypertrophic signaling. Further, we show that calcineurin Aβ associates with mAKAP and that the formation of the mAKAP complex is required for the full activation of the pro-hypertrophic transcription factor NFATc. These data reveal a novel function of the mAKAP complex involving the integration of cAMP and Ca^{2+} signals that promote myocyte hypertrophy.

Key words: mAKAP, Ryanodine receptor, Calcineurin, Hypertrophy, NFATc

Introduction

Myocyte hypertrophy is the compensatory response of the heart to stress and is characterized by non-mitotic growth, increased myofibrillar organization, increased 'fetal' gene expression, and specific changes in ion channel properties (Wilkins and Molkentin, 2004). Maladaptive cardiac hypertrophy can progress to congestive heart failure, a leading cause of morbidity and mortality in the United States. Unfortunately, current therapy for heart failure is generally palliative. A better understanding of the intracellular signal transduction network that controls myocyte cell growth may suggest new therapeutic directions.

Signal transduction networks are composed of second messengers, enzymes, and ion channels that form pathways relaying specific signals from the cell surface to intracellular organelles (Papin et al., 2005). Multiple signaling pathways control the induction of myocyte hypertrophy. These pathways include cAMP-dependent protein kinase A (PKA) pathways, mitogen-activated protein kinase (MAP kinase) pathways, and the calcineurin (CaN)-nuclear factor of activated T-cells (NFATc) transcription factor pathway (Molkentin and Dorn, 2001). An emerging concept in the field of signal transduction is the existence of nodes within a network where multiple signaling pathways converge and share common molecules, thereby facilitating crosstalk between pathways (Papin et al., 2005). Molecules that participate in these centers of integration are of special therapeutic interest, because of the potential for coordinated modulation of multiple signaling pathways.

One protein that is an attractive candidate to coordinate hypertrophic signals elicited from multiple pathways is the 255-kDa scaffold protein mAKAP (muscle A-kinase anchoring protein) (Kapiloff, 2002). mAKAP is located at the nuclear envelope where it is tethered by the integral membrane protein nesprin-1α through the direct binding of spectrin repeats in each protein (Pare et al., 2005). Named for its ability to bind the type II PKA holoenzyme (Kapiloff et al., 1999; Zakhary et al., 2000), mAKAP also binds the cAMP-specific phosphodiesterase PDE4D3, the cardiac-specific type II ryanodine receptor (RyR2), and protein phosphatase 2A (Dodge et al., 2001; Dodge-Kafka et al., 2005; Kapiloff et al., 2001; Marx et al., 2000; Ruehr et al., 2003). Recently, we reported that the MAP kinases MEK5 and ERK5, the small GTPase Rap1, and the cAMP-activated Rap1 exchange factor Epac1 are also anchored by mAKAP (Dodge-Kafka et al., 2005). MEK5 and ERK5 are known to mediate the induction of myocyte hypertrophy by the leukemia inhibitory factor/gp130 receptor (Nicol et al., 2001; Takahashi et al., 2005). Using RNA interference (RNAi) and displacement of mAKAP

from the nuclear envelope, we showed that mAKAP was required for the induction of myocyte hypertrophy by this cytokine-regulated signaling pathway (Dodge-Kafka et al., 2005).

We now report that mAKAP is important for the induction of myocyte hypertrophy by adrenergic receptor signaling. The β-adrenergic receptor stimulates adenylate cyclase and increases intracellular cAMP levels and, subsequently, PKA activity. mAKAP-bound PKA can phosphorylate associated RyR2 (Kapiloff, 2002; Marx et al., 2000; Ruehr et al., 2003), potentiating channel activity (Hain et al., 1995; Wehrens et al., 2004). It is generally understood that RyR2 mediates Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum during excitation-contraction coupling (Fill and Copello, 2002). We have found, however, a small pool of RyR2 associated with mAKAP at the nuclear envelope (Kapiloff et al., 2001). mAKAP-associated RyR2 might regulate the release of Ca^{2+} from adjacent, peri-nuclear sarcoplasmic reticulum or the release of putative stores within the nuclear envelope (Abrenica and Gilchrist, 2000). We now present evidence that mAKAP is integral to the induction of β-adrenergic-stimulated hypertrophy by participating in the CaN-NFATc signaling pathway. We show further that mAKAP is also relevant to α-adrenergic signaling. In contrast to the β-adrenergic receptor, the α-adrenergic receptor activates phospholipase C and phosphatidyl inositol pathways, resulting in increased intracellular Ca^{2+} levels and activation of MAP kinases (including MEK5 and ERK5) and CaN (Dorn and Force, 2005; Nicol et al., 2001). Given the ability of mAKAP to anchor ERK5, PKA, RyR2, and, as shown below, CaN, to the nuclear envelope, we propose that the mAKAP scaffold may serve as an integrator for hypertrophic signaling through the regulation of perinuclear Ca^{2+} and the regulation of gene transcription.

Materials and Methods

Antibodies and antiserum

Commercially available antibodies were as follows: mouse anti-α-actinin (monoclonal EA-53; Sigma), rabbit anti-CaNAβ (Santa Cruz), goat anti-CaNAβ (Santa Cruz), rabbit anti-rat atrial natriuretic factor (ANF; US Biological), mouse anti-myc tag (monoclonal 4A6; Upstate), anti-Flag tag (mouse monoclonal M2 and rabbit polyclonal F7425; Sigma), horseradish peroxidase (HRP)-conjugated anti-His tag (Santa Cruz), HRP-conjugated donkey secondary antibodies (Jackson ImmunoResearch) and Alexa dye-conjugated secondary antibodies (Molecular Probes). mAKAP rabbit antibodies were VO54 anti-mAKAP residues 1406-2314 and VO56 anti-mAKAP residues 1666-2319, as previously described (Kapiloff et al., 1999). mAKAP monoclonal antibody 720 was produced as previously described and binds within amino acid residues 1400-1500 (data not shown) (Pare et al., 2005).

Expression vectors

The small interfering RNA (siRNA) vectors were constructed as follows: An *Eco*RI-*Kpn*I fragment of pSilencer (Ambion) containing the U6 promoter was subcloned into the *Kpn*I and *Mfe*I sites in the pTRE shuttle vector (Adeno-X Tet-Off Expression System, Clontech). Double-stranded hairpin oligonucleotides based upon rat mAKAP mRNA sequence (NCBI GI:5070430, base pairs 7210-7228) were cloned into the *Apa*I and *Eco*RV sites of the pTRE-U6 vector: mAKAP siRNA (sense strand) 5′-GACGAACCTTCCTTCCGAA-TTCAAGAGATTCGGAAGGAAGGTTCGTCTTTTT-3′; control siRNA (sense strand) 5′-GACGAACCCCTGTTCCGAATTCAAGA-GATTCGGAACAGGGGTTCGTCTTTTT-3′. Underlined base pairs in the control siRNA are different from the wild-type (WT) mAKAP siRNA. An oligonucleotide based upon mAKAP base pairs 1043-1061 served as a second control since it was completely ineffective at inhibiting mAKAP expression: 5′-GGTGAACACGGGGAAGAC-GTTCAAGAGACGTCTTCCCCGTGTTCACCTTTTT-3′.

For rescue experiments, a full-length cDNA containing base pairs 440-7065 of the mAKAP rat mRNA was subcloned into pCDNA3.1 (–) MycHis vector (Invitrogen). In order to express a mAKAP form lacking the PKA-binding site (mAKAP Del PKA BD), this plasmid was modified using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). Oligonucleotides were based on the sense sequence, 5′-CAAAAAGATGATGAAGATGGGAGCTCACAGCCTGAAAG-TGAGGTG-3′, deleting base pairs 6284-6346 (codons 2053-2073) of the mAKAP cDNA. Adenovirus containing either the WT or mutant mAKAP cDNA or the U6-promoter siRNA transcription units were constructed using the Adeno-X Tet-Off Expression System (Clontech) and amplified in HEK293 cells according to the manufacturer's recommendations (Kapiloff et al., 2001).

All new plasmids in this study were verified by sequencing of the relevant regions. The Flag-tagged NFATc1 expression vector pSH160c was a gift from Jerry Crabtree (Beals et al., 1997).

Ventricular myocyte culture

Ventricular myocytes (over 90% free of fibroblasts) were prepared from 2-3-day old Sprague-Dawley rats and cultured on Permanox chamber slides (Nunc) coated with 1% gelatin and 0.0015% laminin solution, as previously described (Kapiloff et al., 1999). After 1 day in culture, cells were washed and cultured with Maintenance Medium [79% DMEM, 20% Medium 199, and 1% penicillin/streptomycin solution (P/S, Gibco)]. Myocytes were either infected with adenovirus overnight or transfected for 1 hour with Transfast (Promega, 1-5% transfection efficiency) and then incubated overnight in Maintenance Medium with 4% horse serum. The next day, the medium was replaced with Maintenance Medium containing one or more of the following drugs at standard concentrations: 100 μmol/l phenylephrine (PE) (Thorburn et al., 1997), 1 μM propanolol (Autelitano and Woodcock, 1998), 10 μmol/l isoproterenol (Iso) (Morisco et al., 2001), 100 nmol/l KT5720 (Davies et al., 2000), 400 nmol/l cyclosporin A (CsA) (Molkentin et al., 1998) and 50 μmol/l ryanodine (Ry) (Fill and Copello, 2002).

Immunoprecipitation and back-phosphorylation

For the back-phosphorylation assay, myocytes were stimulated for 30 minutes with 1 mmol/l dibutyryl-cAMP or 10 μmol/l Iso before lysis in immunoprecipitation buffer [50 mmol/l Hepes, pH 7.4, 10% glycerol, 1 mmol/l DTT (dithiothreitol), 1.5 μmol/l pepstatin, 2 μmol/l leupeptin, 1 mmol/l AEBSF (4-(2-aminoethyl)benzenesulphonyl fluoride), 1 mmol/l benzamidine, 5 mmol/l EDTA, 100 mmol/l NaCl, 0.5% Triton X-100, 50 mmol/l sodium fluoride, 1 mmol/l sodium orthovanadate and 10 mmol/l sodium pyrophosphate]. Following immunoprecipitation with 10 μl VO54 anti-mAKAP serum or preimmune serum as previously described (Kapiloff et al., 2001), the immunoprecipitates were resuspended in kinase buffer (50 mmol/l Mops, pH 6.8, 50 mmol/l NaCl, 4 mmol/l $MgCl_2$, 1 mmol/l DTT, 50 nmol/l microcystin-LR) supplemented with 1 μg recombinant PKA C-subunit. Phosphorylation was initiated by addition of 3 μmol/l [γ-^{32}P]ATP (7000 Ci/mmol). After incubation at room temperature for 30 minutes, kinase reactions were stopped by addition of Laemmli sample buffer and 0.25 mmol/l phosphoric acid. Kinase reactions were size-fractionated by SDS-PAGE and transferred to nitrocellulose for autoradiography. The relative extent of phosphorylation was quantified by densitometry and normalized to RyR protein levels detected by immunoblotting.

Co-immunoprecipitations were similarly performed using crude

extracts prepared from rat adult hearts with 10 μg purified mAKAP 720 monoclonal antibody, goat-anti CaNAβ antibody, and the corresponding species-specific non-immune antibodies.

Immunocytochemistry and microscopy

Cell surface measurements

For non-transfected cells, myocytes were cultured at 55,000 myocytes/cm^2. Cells were stained using an antibody to the sarcomeric Z-disk protein α-actinin (in order to distinguish myocytes from contaminating fibroblasts), Alexa fluorescent dye-conjugated specific secondary antibody and Hoechst 33258 DNA stain, as previously described (Kapiloff et al., 2001). Images (100× and 400×) were acquired by digital fluorescence microscopy using a Leica DMRA fluorescence microscope. For each slide, the mean cell area was determined by dividing the aggregate α-actinin-stained area in three micrographs (100×) (measured by counting pixels with Adobe Photoshop 6.0, 0.42 μm^2/pixel^2) by the number of independent myocyte nuclei (~200 per image, binucleated cells counted as one cell).

In parallel, myocytes were co-transfected with mAKAP or control siRNA plasmid, pEGFPN3 green fluorescent protein (GFP)-expression plasmid (Clontech) and, in rescue experiments, pCDNA3.1(–)MycHis mAKAP expression vector. Slides were stained as indicated for exogenous and endogenous mAKAP. GFP-positive cells that showed mAKAP staining comparable to non-transfected cells (for control and rescue cells) or that showed minimal mAKAP staining (siRNA-expressing cells) were analyzed as described above for cell surface area using 400× GFP images.

NFATc1 localization

Myocytes (125,000 myocytes/cm^2) were co-transfected with Flag-tagged NFATc1 expression vector and mAKAP or control siRNA plasmid. Slides were stained with Flag-tag and actinin antibodies. For each slide, at least 25 α-actinin- and Flag-tag-positive cells were examined for NFATc1 localization. The fraction of cells in which NFATc1 immunoreactivity was greater in the nucleus than in the cytoplasm was determined.

ANF expression

Myocytes (125,000 myocytes/cm^2) were infected with adenovirus that constitutively expressed control or mAKAP siRNA, and then stained for mAKAP and ANF expression. For each slide, at least 75 cells were inspected for ANF expression, and the fraction of cells expressing ANF determined.

Leucine Incorporation

A total of 200,000 myocytes (cultured at 55,000 myocytes/cm^2) were infected with adenovirus and, after 36 hours of culture in Maintenance Medium, were cultured for 18 hours in the presence of 2 μCi [4,5-^{3}H]leucine, 20 μmol/l arabinosylcytosine, and agonist as indicated. Total TCA precipitable tritiated protein was determined as previously described (Takahashi et al., 2005). Parallel, control cultures were used to monitor mAKAP expression by immunoblot of total cell extracts (Kapiloff et al., 2001).

RIIα-overlay assay

COS-7 cells were cultured and infected with adenovirus expressing either WT mAKAP or mAKAP Del PKA BD as previously described (Kapiloff et al., 2001). Following immunoprecipitation using affinity-purified VO54 antibody, the mAKAP protein was analyzed by RIIα-overlay using 10 nmol/l His-tagged, recombinant PKA RIIα-subunit and 1:10000 HRP-conjugated anti-His antibody (Kapiloff et al., 1999). The identical blot was analyzed by immunoblotting with VO54 antibody to show equal loading.

Statistics

For the above described experiments, all numerical data are presented as ± s.e.m. *P* values were determined using a two-tailed, paired or unpaired Student's *t*-test, as appropriate. Single factor analysis of variance (ANOVA) was performed as indicated.

Results

Regulation of mAKAP-bound RyR2s in cardiac myocytes

It has been reported that the majority of RyR2 at the sarcoplasmic reticulum is phosphorylated by PKA on Ser-2809 following β-adrenergic stimulation (Wehrens and Marks,

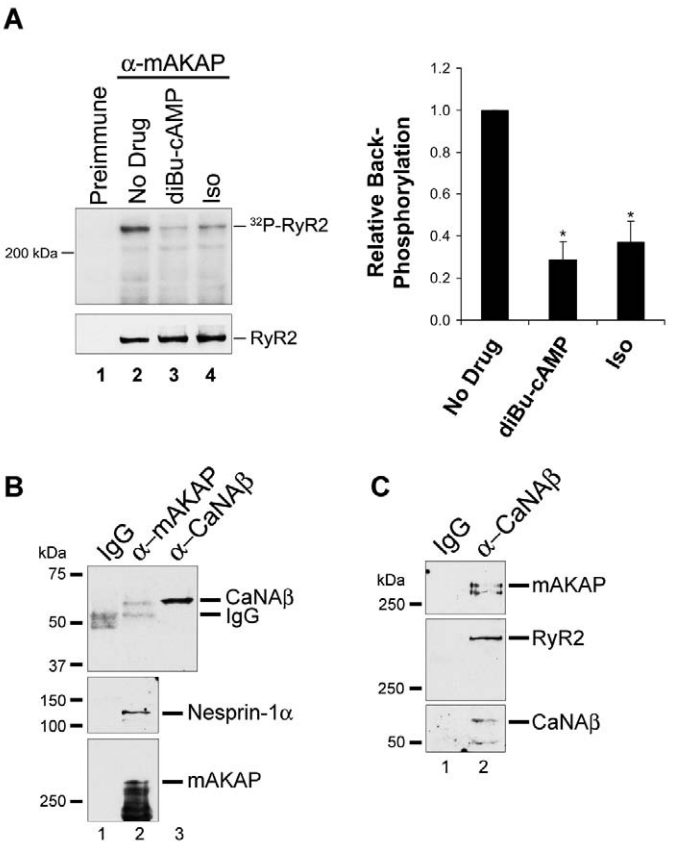

Fig. 1. The mAKAP complex includes the PKA substrate RyR2 and CaNAβ. (A) Myocyte cultures were treated for 30 minutes with 1 mmol/l diBu-cAMP or 10 μmol/l Iso. mAKAP-associated RyR2 co-immunoprecipitated with VO54 mAKAP serum (lanes 2-4), but not preimmune control serum (lane 1). The immunoprecipitates were back-phosphorylated with recombinant PKA catalytic subunit and [γ-^{32}P]ATP (top panel). RyR2 was detected by immunoblotting with a RyR2 monoclonal antibody (bottom panel). The bar chart shows relative back-phosphorylation (mean ± s.e.m.). *P<0.003 compared to control; n=5; ANOVA, P<0.001. (B) Immune complexes were precipitated from adult rat heart extracts with non-specific mouse IgG (lane 1), mAKAP monoclonal antibody 720 (lane 2), and goat anti-CaNAβ (lane 3). CaNAβ (top panel), nesprin-1α (middle panel) and mAKAP (bottom panel) were detected by immunoblotting. Relative molecular mass markers are indicated. (C) Immune complexes were precipitated from adult rat heart extracts with non-specific goat IgG (lane 1) and goat anti-CaNAβ antibody (lane 2). mAKAP (top panel), RyR2 (middle panel) and CaNAβ (bottom panel) were detected by immunoblotting. n=3.

2004). We were interested in establishing whether peri-nuclear RyR2 bound to mAKAP was similarly regulated. The phosphorylation of mAKAP-bound RyR2 was measured by the PKA back-phosphorylation assay (Marx et al., 2000) (Fig. 1A). In this in vitro assay, decreased back-phosphorylation with recombinant PKA C-subunit is indicative of increased phosphorylation by endogenous PKA in vivo. Following the treatment of primary neonatal rat ventricular myocyte cultures for 30 minutes with the β-adrenergic agonist isoproterenol (Iso) or the cell-permeable cAMP analog dibutyryl-cAMP (diBu-cAMP), mAKAP-bound RyR2s were assayed by back-phosphorylation (Fig. 1A). Treatment with Iso and diBu-cAMP resulted in the in vivo phosphorylation of approximately 63% and 71% of the available PKA sites on mAKAP-bound RyR2s, respectively (Fig. 1A, graph). These data demonstrate that β-adrenergic stimulation can induce the phosphorylation of mAKAP-associated RyR by PKA.

Association of CaNAβ with the mAKAP complex

Given the function of the mAKAP complex in facilitating RyR2 phosphorylation by PKA and the involvement of the complex in cytokine-induced hypertrophy (Dodge-Kafka et al., 2005), we were interested in determining whether CaN, a Ca^{2+}-dependent enzyme also involved in hypertrophic signaling (Bueno et al., 2002), was associated with the mAKAP complex. mAKAP complexes were immunoprecipitated from crude adult rat heart extracts and blotted for the presence of CaNAβ (Fig. 1B). Significantly, CaNAβ was detected in mAKAP immunoprecipitates, but not in IgG controls (Fig. 1B, upper panel). As positive controls, both mAKAP and the mAKAP nuclear envelope receptor nesprin-1α were detected in the immunoprecipitates (Fig. 1B, lower two panels). Reciprocally, CaNAβ immunoprecipitates from heart extract contained mAKAP and RyR2 (Fig. 1C, upper two panels, lane 2). None of the proteins were detected in IgG-negative controls (Fig. 1C, lane 1). Similar results were obtained using extracts derived from neonatal myocyte cultures (data not shown). These results confirmed that both CaNAβ and RyR2 exist in a complex with mAKAP.

Role of the RyR2 and CaN in β-adrenergic-induced hypertrophy

Having established that Iso could induce the phosphorylation of mAKAP-associated RyR2 and that CaNAβ could co-immunoprecipitate with mAKAP, we tested whether RyR2 and CaN were involved in Iso-induced myocyte hypertrophy. Primary myocyte monolayer cultures were maintained for 2 days in minimal medium with or without Iso, the RyR inhibitor ryanodine (Ry) or the CaN inhibitor cyclosporin A (CsA; Fig. 2). As expected, Iso promoted symmetric growth of the myocyte and myofibrillar organization (Fig. 2Ad) (Schaub et al., 1997). In the absence of inhibitors, Iso-treated cells had 30% more actinin-stained surface area than control cells (1127±29 μm^2 vs 866±72 μm^2; Fig. 2B, bars 1 and 4). Inhibition of both RyR2 (963±65 μm^2, bar 5) and CaN (982±48 μm^2, bar 6) activities attenuated the Iso-induced cellular growth and decreased myofibrillar organization (Fig. 2Ae,f). As a control, neither inhibitor significantly affected myocyte size in the absence of Iso. These results demonstrate that RyR and CaN activities are important for the induction of cellular hypertrophy by β-adrenergic receptor signaling.

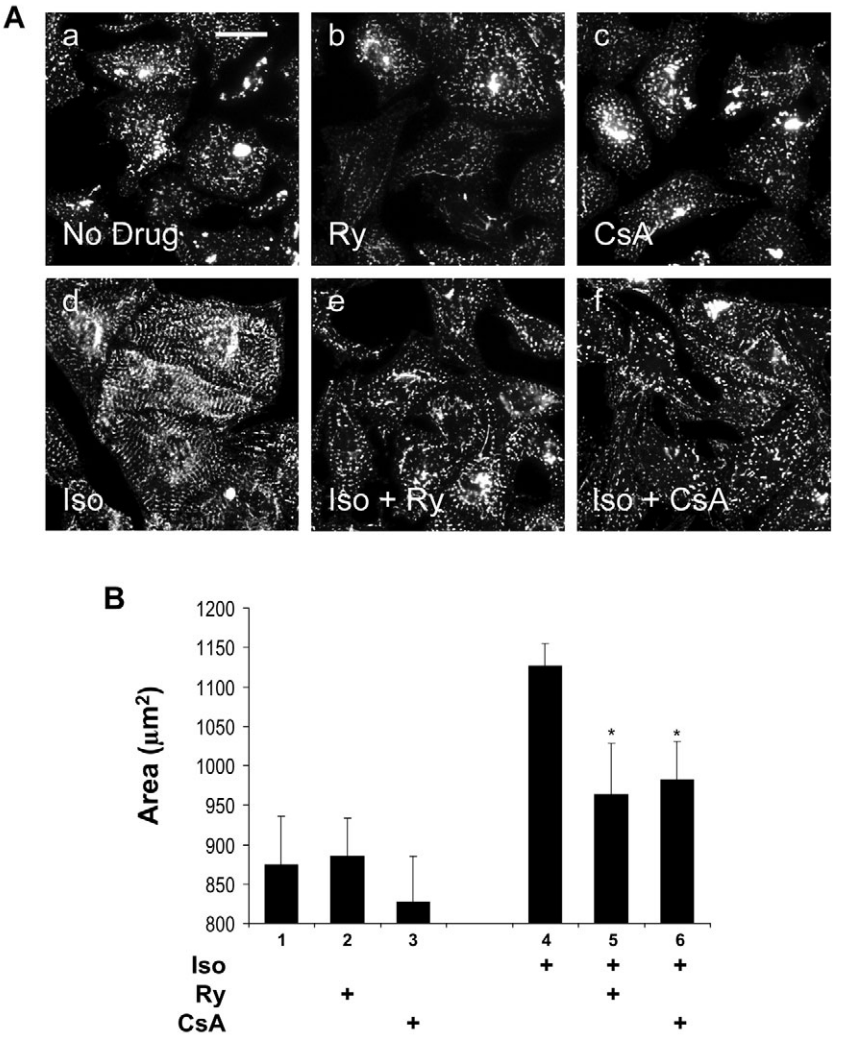

Fig. 2. Ryanodine receptor and CaN activities are required for induction of cardiac myocyte hypertrophy by isoproterenol. (A) Primary myocytes were cultured for 2 days in a minimal medium containing either no drug (a), 10 μmol/l Iso (d-f), 50 μmol/l Ry (b,e) and/or 400 nmol/l CsA (c,f). Cells were fixed and stained with antibody for the sarcomeric Z-disk protein α-actinin (grayscale). Scale bar in a indicates 20 μm. (B) Mean cell surface area (± s.e.m.). *P<0.04 relative to the value for cells treated with Iso alone; $n \geqslant 3$ independent myocyte preparations; ANOVA, P=0.01.

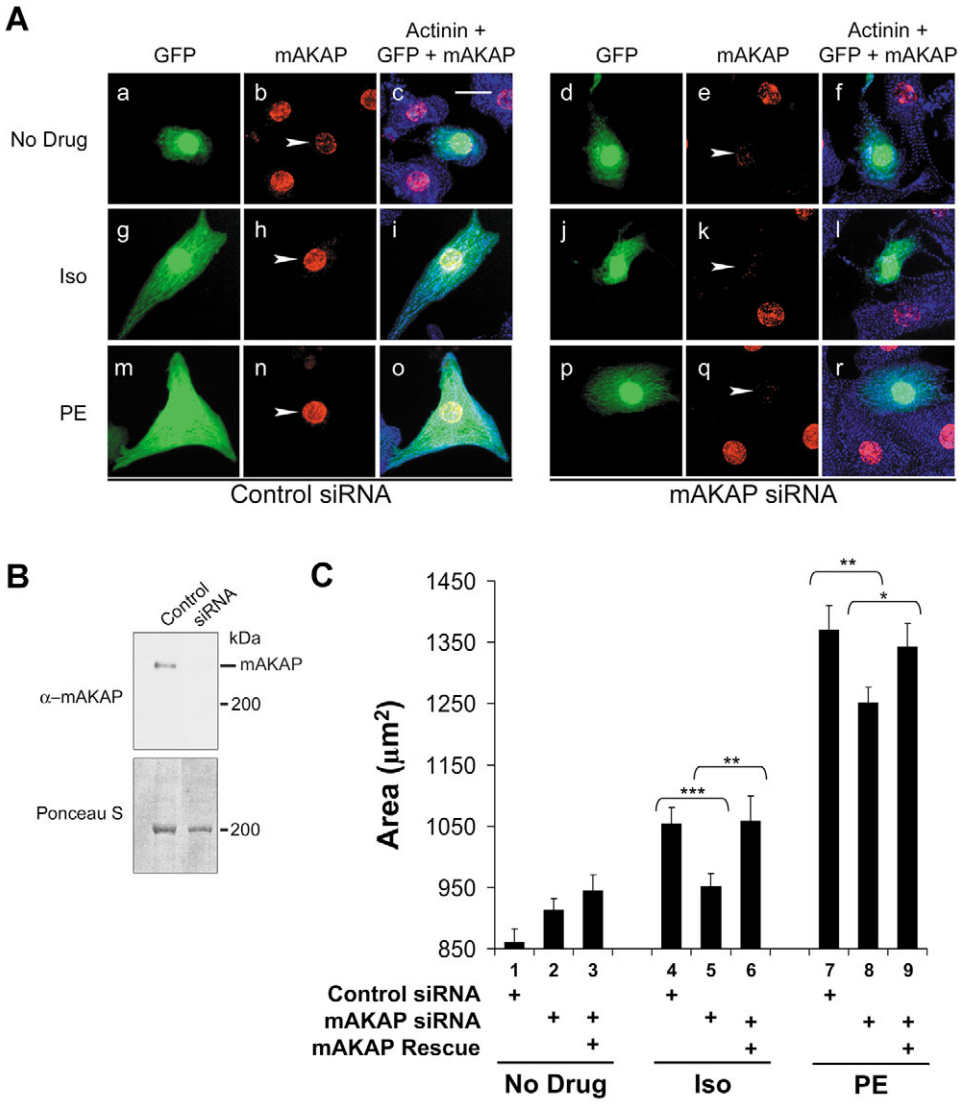

Fig. 3. mAKAP is involved in the induction of adrenergic-stimulated myocyte hypertrophy. (A) Following co-transfection with a GFP expression vector and an expression vector for either control (left panels) or mAKAP siRNA (right panels), primary myocytes were cultured for 2 days in minimal medium containing either no drug (a-f), 10 μmol/l Iso (g-l), or 100 μmol/l PE (m-r). Cells were stained with mAKAP VO56 (red, b,e,h,k,n,q) and α-actinin (blue, c,f,i,l,o,r) antibodies. The nuclei of transfected, GFP-expressing myocytes (green) are indicated with arrowheads on the mAKAP panels. Bar, 20 μm. (B) The level of mAKAP expression in myocytes infected with control or mAKAP siRNA-expressing adenoviruses were assayed by immunoblotting with VO56 mAKAP antibody. Total protein was detected by Ponceau S stain; the major band is myosin. (n=3) (C) Mean cell surface area (± s.e.m.) for GFP-labeled cells co-expressing either the control siRNA, the mAKAP siRNA, or the mAKAP siRNA and exogenous, myc-tagged mAKAP protein (mAKAP rescue). *P=0.04, **P=0.01, ***P=0.003 for the samples compared, n>150 with myocytes derived from ⩾7 independent cultures; ANOVA, P=0.004 and 0.02 for Iso- and PE-treated data, respectively.

mAKAP siRNA inhibits adrenergic-induced hypertrophy

The requirement for mAKAP in myocyte hypertrophy was studied by RNAi using an expression plasmid for a siRNA based on rat mAKAP mRNA 3′ non-coding sequence (Dodge-Kafka et al., 2005). Myocytes were co-transfected with either a mAKAP or a control siRNA expression plasmid and a marker GFP expression plasmid. Successful RNAi was evident by the reduction in mAKAP staining at the nuclear envelope in cells co-expressing GFP and the mAKAP siRNA (Fig. 3A, right panels). In contrast, the mAKAP-nuclear envelope staining was detected in both non-transfected cells and cells co-expressing GFP and the control siRNA (left panels). As expected, myocyte infection with mAKAP siRNA adenovirus decreased mAKAP protein levels by ~80% compared to control siRNA (Fig. 3B).

To test whether mAKAP RNAi could inhibit β-adrenergic-induced hypertrophy, we measured the cell surface area, i.e. the area showing GFP fluorescence, of Iso-treated, transfected myocytes. Iso induced a 23% increase in surface area of control siRNA cells (860±22 μm^2 vs 1054±26 μm^2; Fig. 3C, bars 1 and 4). RNAi of mAKAP expression inhibited the Iso-induced cellular growth, such that cells were only 4% larger than mAKAP siRNA-expressing cells cultured without agonist (913±19 μm^2 vs 952±21 μm^2, bars 2 and 5). The effect of mAKAP RNAi was specific to agonist-induced hypertrophy, given that there was no significant difference in size between mAKAP and control siRNA-expressing myocytes cultured without drug (bars 1 and 2). In order to demonstrate the specificity of the mAKAP siRNA, we performed rescue experiments with an expression plasmid containing a mAKAP cDNA lacking the siRNA target sequence (bars 3 and 6). Iso-treated mAKAP-siRNA-expressing cells rescued by recombinant mAKAP expression were comparable in size (1058±40 μm^2) to those expressing the control siRNA (Fig. 3C, bars 4 and 6).

To extend these findings, we assessed the effect of mAKAP RNAi on the induction of hypertrophy by the α-adrenergic agonist PE, which can strongly induce myocyte hypertrophy in a CaN-dependent manner (Molkentin et al., 1998). PE promoted a 59% increase in the surface area of control siRNA-expressing cells (860±22 μm^2 vs 1370±40 μm^2; Fig. 3C, bars 1 and 7). RNAi of mAKAP expression inhibited the PE-induced cellular growth, such that cells were only 37% larger

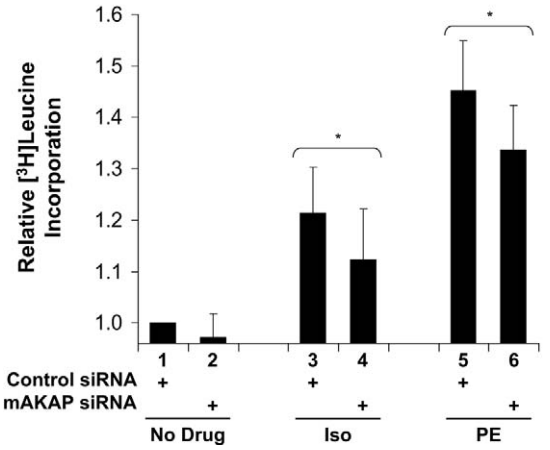

Fig. 4. mAKAP RNAi inhibits protein synthesis. To detect new protein synthesis, myocytes were infected with control or mAKAP siRNA-expressing adenovirus. Myocytes were cultured for 18 hours in medium containing [^{3}H]leucine and either no agonist (No Drug), 10 μmol/l Iso, or 100 μmol/l PE and 1 μmol/l propanolol (PE). Total [^{3}H]leucine incorporation was determined. Data are normalized to the control siRNA, untreated sample (bar 1). *$P \leqslant 0.05$; $n \leqslant 4$ independent myocyte preparations.

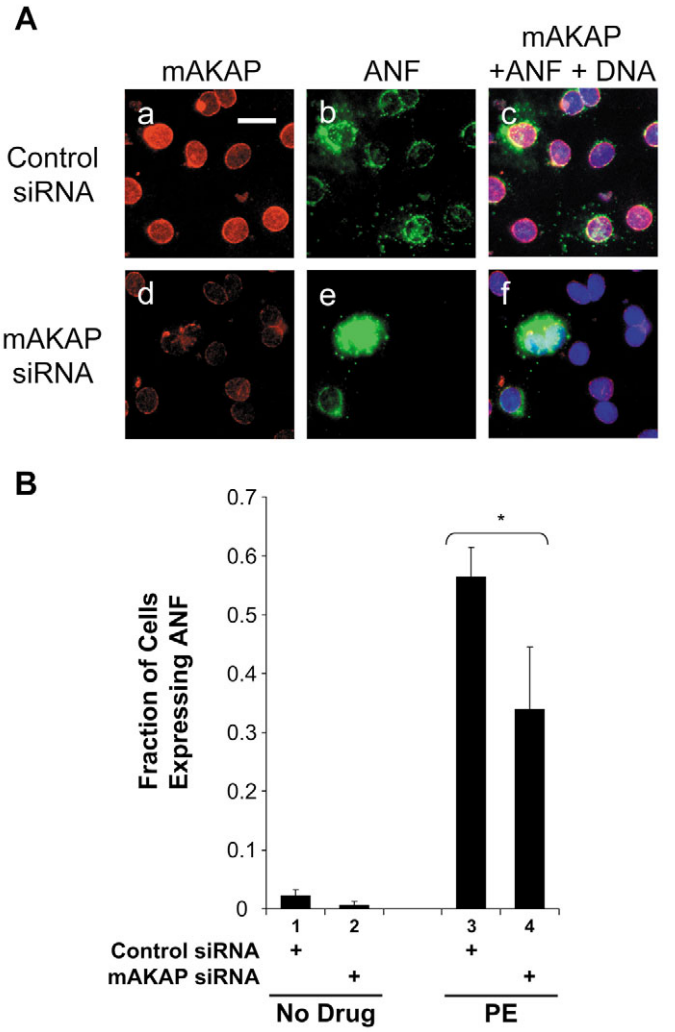

Fig. 5. mAKAP RNAi inhibits ANF expression. (A) Myocytes were infected with control (a-c) or mAKAP (d-f) siRNA-expressing adenovirus. The cells were treated with 100 μmol/l PE and 1 μmol/l propanolol for 48 hours (a-f) or left untreated (not shown), and then stained with monoclonal mAKAP antibody (red, a,d), rabbit ANF antibody (green, b,e) and Hoechst DNA stain (shown in the merged images in c,f). Bar, 20 μm. (B) The fraction of myocytes showing peri-nuclear, Golgi-type ANF staining is indicated for treated and non-treated myocytes. Data are average expression ± s.e.m. *P=0.03; n=4.

than mAKAP siRNA-expressing cells cultured without agonist (913±19 μm^2 vs 1251±25 μm^2, bars 2 and 8). Moreover, rescue of mAKAP expression restored PE-induced cellular growth (1343±37 μm^2, bar 9).

In addition to cell surface measurements, we assayed two other established markers for cardiac hypertrophy, protein synthesis (Fig. 4) and ANF expression (Fig. 5), using adenovirus-infected myocytes. [^{3}H]leucine incorporation into newly synthesized protein was 21±9% and 45±10% greater in Iso- and PE-stimulated, control siRNA-expressing myocytes than in the absence of agonist, respectively (Fig. 4, bars 1, 3 and 5). mAKAP RNAi specifically reduced Iso and PE-induced [^{3}H]leucine incorporation to 12±10% and 34±9% over baseline (bars 4 and 6). In accord with the above data, in control siRNA-expressing cells, PE induced ANF expression in 0.56±0.05 of the infected myocytes (Fig. 5A,B, bars 1 and 3). Depletion of mAKAP expression limited ANF expression to 0.34±0.11 of the stimulated myocytes (bar 4). Taken together, these data support the hypothesis that the mAKAP signaling complex participates in adrenergic-induced cellular hypertrophy.

PKA binding to mAKAP is important for hypertrophic signaling

In order to test whether mAKAP-anchored PKA is necessary for hypertrophic signaling, we generated a mutant mAKAP form lacking amino acid residues 2053-2073 comprising the PKA binding domain (Kapiloff et al., 1999) (mAKAP Del PKA BD). We verified that the mutant protein did not bind PKA by PKA RIIα-subunit overlay assay (Fig. 6D). The effect of this mutant on myocyte surface area was compared to that of WT by transient transfection (Fig. 6A-D). Because the exogenous mAKAP proteins were myc-tagged, we were able to verify their expression by staining the transfected myocytes with myc-tag antibody (Fig. 6A-Cf,j). In order to avoid confounding, endogenous mAKAP, the myocytes were also co-transfected with the mAKAP siRNA expression plasmid. Neither the mAKAP WT nor the mAKAP Del PKA BD cDNA contained the siRNA target sequence found in the 3′ non-coding region of the endogenous mAKAP mRNA. By double-staining the myocytes with mAKAP antibody, we were able to selectively study cells that expressed recombinant mAKAP at approximately the same level as adjacent, non-transfected cells expressing endogenous mAKAP (Fig. 6A-Cg,k). Agonist-treated cells expressing mAKAP WT were significantly larger than cells expressing mAKAP Del PKA BD or cells lacking mAKAP altogether (Fig. 6E). In this experiment, Iso- and PE-treated cells expressing mAKAP WT were 19% and 53% larger than non-treated cells, respectively (Fig. 6E, bars 2, 5 and 8). By contrast, Iso- and PE-treated cells expressing mAKAP Del PKA BD were 6% and 38% larger than non-treated cells,

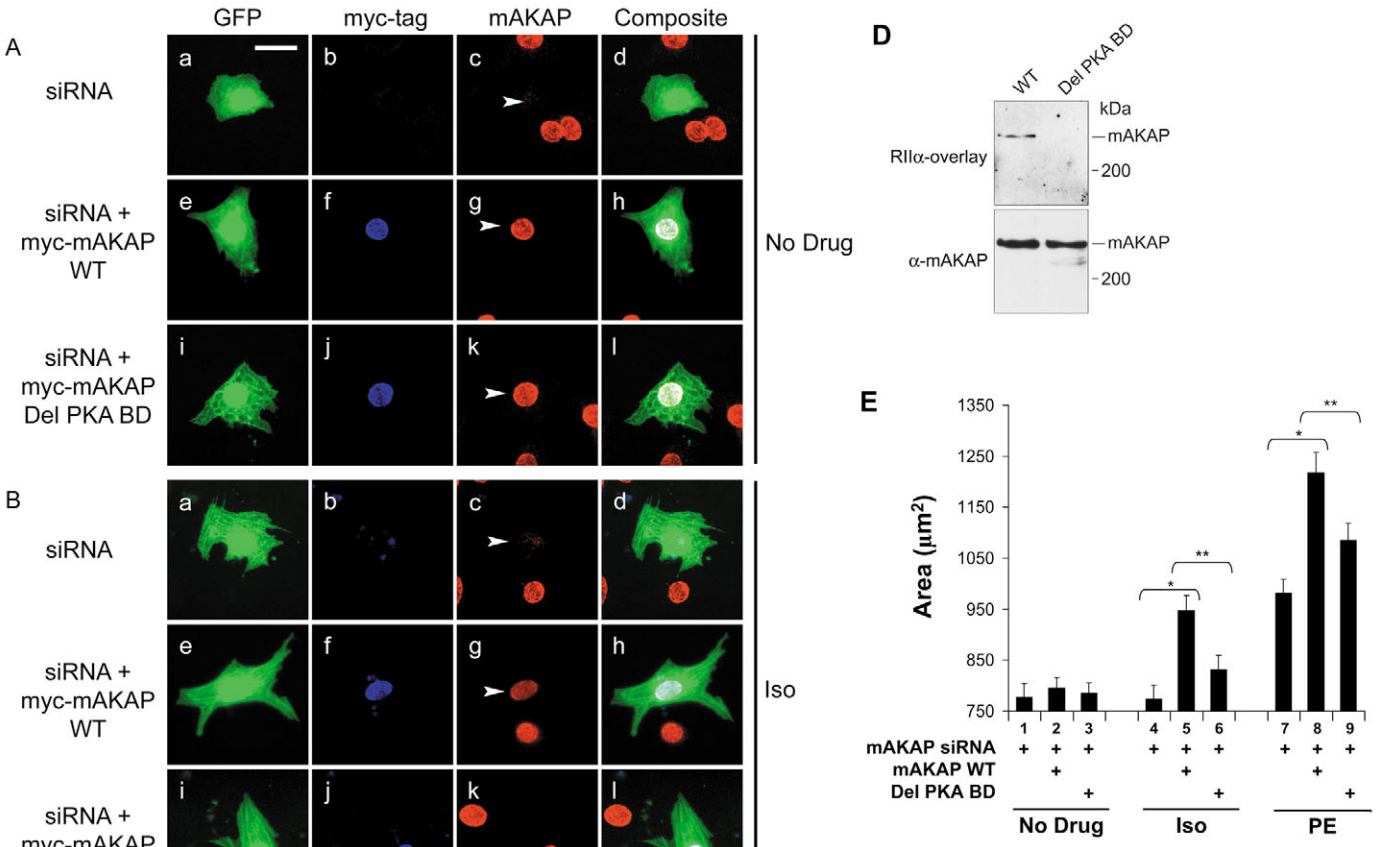

Fig. 6. mAKAP-bound PKA is important for the transduction of signals that induce cardiac myocyte hypertrophy. (A-C) Following co-transfection with expression vectors for GFP and mAKAP siRNA (a-d), primary myocytes were cultured for 2 days in minimal medium containing either no drug (A), 10 μmol/l Iso (B), or 100 μmol/l PE (C). Additional myocytes were co-transfected with a third expression plasmid for either myc-tagged mAKAP WT (e-h) or mAKAP Del PKA BD (i-l). Cells were stained with myc-tag (blue, b,f,j) and mAKAP VO56 (red, c,g,k) antibodies. The nuclei of transfected, GFP-expressing myocytes (green) are marked with arrowheads on the mAKAP and myc panels. Bar, 20 μm. (D) mAKAP WT and mAKAP Del PKA BD protein were expressed by adenoviral infection of COS-7 cells and purified by immunoprecipitation. PKA binding activity of the WT and mutant protein was assayed by RIIα overlay assay using recombinant His-tagged RIIα protein and HRP-conjugated anti-His antibody. Equal loading was demonstrated by immunoblotting the same filter with VO54 mAKAP antibody (n=3). (E) Mean cell surface area (±s.e.m.) for myocytes cultured as in A. *P<0.015, **P<0.0001 for the samples compared; n>115 from ⩾6 independent cultures; ANOVA, $P<10^{-4}$ for the entire data set, and Iso- and PE-treated data alone.

respectively (Fig. 6E, bars 3, 6 and 9). Thus, mAKAP-anchored PKA plays a role in the induction of hypertrophy.

mAKAP and NFATc1 activation

An established substrate for mAKAP-bound PKA is RyR2. We proposed that mAKAP-associated RyR2 mediates local Ca^{2+} release that might regulate CaNAβ in the mAKAP complex. In order to determine whether the mAKAP complex is relevant to CaN activity in cells, we assayed the activation of the CaN substrate NFATc1 (Fig. 7A). Since commercially available antibodies for the NFATc family members do not adequately detect endogenous NFATc protein by immunocytochemistry, flag-tagged NFATc1 was used as a representative of the NFATc transcription factor family. In the absence of stimulus, NFATc transcription factor family members are highly phosphorylated and sequestered in the cytoplasm (Crabtree and Olson, 2002). Following de-phosphorylation by CaN, nuclear localization

signals within NFATc family members are exposed, and the transcription factor is imported into the nucleus. Under basal conditions, expressed flag-tagged NFATc1 was located primarily in the cytosol of cultured myocytes (0.26±0.10; Fig. 7B, bars 1 and 2). In contrast, in the majority of myocytes treated with Iso and PE, NFATc1 was enriched in the nucleus (0.69±0.10 and 0.92±0.02, respectively; Fig. 7B, bars 3 and 7). As is well established (Crabtree and Olson, 2002), addition of the CaN inhibitor CsA prevented the nuclear translocation of NFATc1 regardless of the agonist (Iso, 0.09±0.03; PE, 0.07±0.02; Fig. 7B, bars 4 and 8). In order to establish a role for PKA in NFATc transcription factor activation, myocytes were treated with the PKA inhibitor KT5720. This treatment inhibited NFATc1 nuclear translocation for both agonists (Iso, 0.15±0.05; PE, 0.67±0.11, bars 5 and 9). To demonstrate that the mAKAP complex was specifically involved in NFATc1 translocation, we used RNAi (Fig. 7A). NFATc1 nuclear localization was significantly decreased by mAKAP RNAi for both agonists (Iso, 0.43±0.07; PE, 0.74±0.08; Fig. 7B, bars 6 and 10). Thus, the presence of the scaffold mAKAP facilitates activation of the NFATc transcription factor.

Discussion

In this manuscript we provide evidence that mAKAP participates in the activation of the Ca^{2+}/calmodulin-dependent CaN/NFATc transcription factor pathway and in the induction of myocyte hypertrophy by adrenergic agonists. In particular, we propose the following model for mAKAP signaling following β-adrenergic receptor activation: β-adrenergic stimulation increases local cAMP levels that promote PKA-catalyzed phosphorylation of perinuclear RyR2 channels associated with mAKAP. Increased channel activity caused by RyR2 phosphorylation may result in the continued release of Ca^{2+} from stores in that vicinity and a persistent, elevated local Ca^{2+} concentration. One function of the elevated Ca^{2+} levels may be the maintenance of high local levels of mAKAP-associated CaNAβ activity. CaNAβ may in turn dephosphorylate the substrate NFATc transcription factor, promoting nuclear translocation and hypertrophic gene expression (Crabtree and Olson, 2002; Timmerman et al., 1996).

Prior and current evidence lend support to our model. Our previously published work showed that mAKAP-bound PKA phosphorylates associated RyR2 in vitro (Kapiloff et al., 2001). In this paper, we show that mAKAP-associated RyR2 is phosphorylated by PKA following β-adrenergic stimulation in vivo (Fig. 1). Interestingly, PKA-catalyzed RyR2 Ser-2809 phosphorylation is thought to be induced by elevated circulating catecholamines during the development of human heart failure, and has been detected in several animal models of cardiac failure (Wehrens and Marks, 2004). This phosphorylation event has been associated with increased channel opening and the presence of leaky, subconductance currents (Wehrens et al., 2004).

As suggested by our model, a potential target for Ca^{2+} released by mAKAP-associated RyR2 may be mAKAP-associated CaNAβ (Fig. 1B). NFATc transcription factors are regulated by cytoplasmic-nuclear shuttling (Wilkins and Molkentin, 2004). Inactive NFATc factors are sequestered in the cytosol in a hyperphosphorylated state. CaN dephosphorylation activates NFATc, resulting in nuclear translocation and the promotion of gene expression. Activated NFATc can be rephosphorylated by several cytosolic and nuclear protein kinases, including GSK-3β. Peri-nuclear CaNAβ may affect the equilibrium between NFATc dephosphorylation and phosphorylation, favoring the accumulation of dephosphorylated NFATc in the nucleus when the mAKAP complex is activated. In support of this hypothesis, we showed that mAKAP depletion inhibited

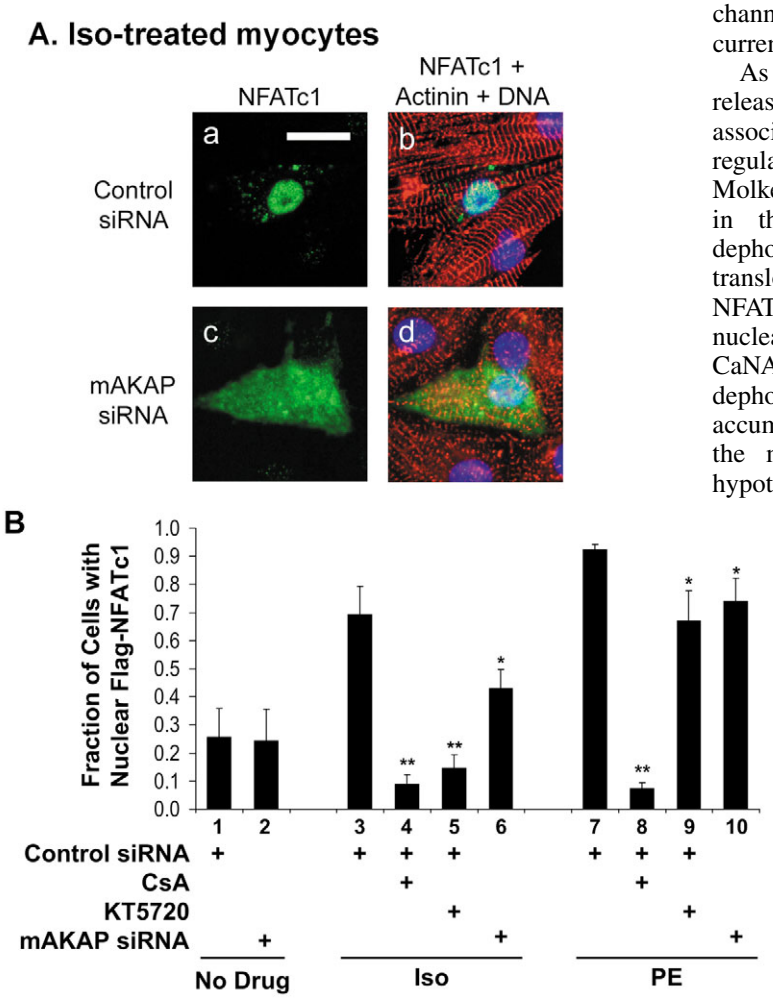

Fig. 7. The mAKAP complex contributes to NFATc1 activation. (A) Primary myocytes were co-transfected with an expression vector for Flag-tagged NFATc1 and either the control (a,b) or mAKAP (c,d) siRNA expression plasmid. The myocytes were treated for 2 days with no drug, 10 μmol/l Iso, 100 μmol/l PE, 400 nmol/l CsA and/or 100 nmol/l KT5720. Representative cells cultured in Iso-containing medium are shown. Cells were fixed and stained with antibodies for the Flag-tag (green, a,c) and α-actinin (red, b,d) and with Hoechst 33258 DNA stain (blue, b,d). Bar, 20 μm. (B) The fraction of transfected myocytes with predominately nuclear NFATc1 (mean±s.e.m.). *P<0.05, **P<0.002 in comparison to the similarly treated control siRNA sample, $n \geq 6$; ANOVA, $P<10^{-4}$ for entire data set and Iso- and PE-treated data alone.

adrenergic-induced NFATc1 translocation in cultured myocytes (Fig. 7).

CaNAβ and NFATc family members are required for the induction of pathologic hypertrophy (Wilkins and Molkentin, 2004). Using RNAi, we showed that the mAKAP scaffold is also important for induction of myocyte hypertrophy. Moreover, by expression of a mutant form of mAKAP lacking the PKA binding domain (mAKAP Del PKA BD), we showed that the ability of mAKAP to bind PKA was functionally relevant to mAKAP complex hypertrophic signaling. The PKA responsible for phosphorylation of mAKAP-associated RyR2 is presumably PKA bound to mAKAP (Kapiloff et al., 2001; Marx et al., 2000; Ruehr et al., 2003). While PKA can diffuse non-specifically towards its substrates, targeting by AKAPs increases the rate of the phosphorylation reaction and the efficiency of the signaling (Kapiloff, 2002). While the expression of exogenous myc-tagged mAKAP WT was able to reverse the inhibition of myocyte hypertrophy by the mAKAP siRNA, expression of mAKAP Del PKA BD only partially rescued this phenotype (Fig. 6). There are more than a dozen AKAPs in the myocyte and at least two, mAKAP and AKAP 149, are known to be present at the nuclear envelope (Kapiloff, 2002). These results demonstrate that regardless of other perinuclear AKAPs that may also bring PKA to the nuclear envelope, the targeting of PKA by the mAKAP scaffold to its specific spatial micro-domain is important for the transduction of pro-hypertrophic signaling.

mAKAP was required not only for β-adrenergic, but also for α-adrenergic signaling, albeit to a quantitatively lesser degree. Because the α-adrenergic receptor acts through phospholipase C and phosphatidyl inositol pathways to stimulate Ca^{2+} release and CaN (Dorn and Force, 2005), a role for the mAKAP complex and PKA in α-adrenergic signaling was paradoxical. However, PE can also activate ERK5, another mAKAP-binding partner involved in hypertrophic signaling (Dodge-Kafka et al., 2005; Nicol et al., 2001). While ERK5 can directly regulate pro-hypertrophic transcription factors, ERK5 bound to mAKAP will also inhibit associated PDE4D3 cAMP phosphodiesterase activity, resulting in increased mAKAP-bound PKA activity (Dodge-Kafka et al., 2005). Because PDE4 is responsible for maintaining the basal level of cAMP in resting myocytes (Mongillo et al., 2004), PDE4D3 inhibition by ERK5 may explain how PE can signal through PKA in the mAKAP complex (Fig. 6).

In this work we have begun to define a role for the mAKAP complex in adrenergic-induced cardiac hypertrophy. We recently found that mAKAP is critical to leukemia inhibitory factor/gp130 receptor-stimulated hypertrophy (Dodge-Kafka et al., 2005). Together, these results suggest that the mAKAP complex is involved in the overall regulation of myocyte cell growth in pathologic states. The association with multiple signaling molecules sensitive to different upstream stimuli, i.e. PKA (Kapiloff et al., 1999), Epac1 (Dodge-Kafka et al., 2005), MEK5 (Dodge-Kafka et al., 2005), rap1 (Dodge-Kafka et al., 2005), RyR2 (Kapiloff et al., 2001; Marx et al., 2000) and CaNAβ, establishes mAKAP as a significant coordinator of the cardiac myocyte signaling network (Papin et al., 2005). Interestingly, it has recently been reported that CaNA, NFATc, MEK1 and ERK1 associate in a complex (Sanna et al., 2005; Yang et al., 2005). NFATc3 and NFATc4 phosphorylation by ERK1 or its substrate RSK can increase the DNA binding affinity of the transcription factor. Although it remains to be established whether each of the mAKAP binding partners can be simultaneously present in the same mAKAP complex, the potential for crosstalk by the different signaling pathways through the mAKAP complex is intriguing and will be the subject of future investigations. Meanwhile, the identification of mAKAP as a central hypertrophic signaling scaffold makes it an attractive target for therapeutic invention in the treatment of pathologic cardiac hypertrophy.

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