Published in final edited form as:

J Mol Biol. 2013 September 23; 425(18): . doi:10.1016/j.jmb.2013.06.014.

Protein Kinase A and Phosphodiesterase-4D3 Binding to Coding Polymorphisms of Cardiac Muscle Anchoring Protein (mAKAP)

Abeer Rababa'h¹, John W. Craft Jr², Cori S. Wijaya¹, Fatin Atrooz¹, Qiying Fan¹, Sonal Singh¹, Ashley N. Guillory¹, Panagiotis Katsonis³, Olivier Lichtarge³, and Bradley K. McConnell¹

¹Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Texas Medical Center, Houston, TX 77204, USA

²Department of Biology and Biochemistry, University of Houston, Texas Medical Center, Houston TX 77204, USA

³Department of Molecular and Human Genetics, Baylor College of Medicine, Texas Medical Center, Houston TX 77030, USA

Abstract

Protein kinase A (PKA) substrate phosphorylation is facilitated through its co-localization with its signaling partner by A-kinase anchoring proteins (AKAPs). mAKAP (muscle-selective AKAP) localizes PKA and its substrates such as phosphodiesterase-4D3 (PDE4D3), ryanodine receptor, and protein phosphatase 2A (PP2A) to the sarcoplasmic reticulum and perinuclear space. The genetic role of mAKAP, in modulating PKA/PDE4D3 molecular signaling during cardiac diseases, remains unclear. The purpose of this study was to examine the effects of naturally occurring mutations in human mAKAP on PKA and PDE4D3 signaling. We have recently identified potentially important human mAKAP coding non-synonymous polymorphisms located within or near key protein binding sites critical to β -adrenergic receptor signaling. Three mutations (P1400S, S2195F, and L717V) were cloned and transfected into a mammalian cell line for the purpose of comparing whether those substitutions disrupt mAKAP binding to PKA or PDE4D3. Immunoprecipitation study of mAKAP-P1400S, a mutation located in the mAKAP-PDE4D3 binding site, displayed a significant reduction in binding to PDE4D3, with no significant changes in PKA binding or PKA activity. Conversely, mAKAP-S2195F, a mutation located in mAKAP-PP2A binding site, showed significant increase in both binding propensity to PKA and PKA activity. Additionally, mAKAP-L717V, a mutation flanking the mAKAP-spectrin repeat domain, exhibited a significant increase in PKA binding compared to wild type, but there was no change in PKA activity. We also demonstrate specific binding of wild-type mAKAP to PDE4D3. Binding results were demonstrated using immunoprecipitation and confirmed with surface plasmon resonance (Biacore-2000); functional results were demonstrated using activity assays, Ca^{2+} measurements, and Western blot. Comparative analysis of the binding responses of mutations to mAKAP could provide important information about how these mutations modulate signaling.

Keywords

β -adrenergic receptor; hypertrophy; PDE4D3; surface plasmon resonance; immunoprecipitation

© 2013 Elsevier Ltd. All rights reserved.

Correspondence to Bradley K. McConnell: 4800 Calhoun Road, Science and Research Building 2, Room 460, Houston, TX 77024, USA. bkmcconn@central.uh.edu.

Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jmb.2013.06.014>

Introduction

Protein kinase A (PKA) phosphorylation is central to the regulation of many cellular processes including contraction, metabolism, ion fluxes, and gene expression in myocytes [1]. The specificity of PKA signaling is mediated in part by binding of PKA to Akinase anchoring proteins (AKAPs) that are targeted to specific intracellular locations with individual substrates, where it is activated locally by cAMP [2,3]. More than 70 different AKAPs have been identified in a variety of cell types and at least 14 AKAPs have been shown to be expressed in rodent and human hearts. All AKAPs bind PKA through a conserved hydrophobic amphipathic α -helix anchoring domain, which interacts with an N-terminal dimerization and docking (D/D) hydrophobic groove of the PKA regulatory type II (RII) subunit dimer [4]. AKAPs also have a unique targeting sequence, which positions the AKAP complex in close proximity to its substrate, as well as binding sites for other signaling molecules [2,5].

In addition to PKA, AKAPs can bind other protein kinases, cAMP-dependent phosphodiesterases (PDEs), protein phosphatase, and substrate proteins of PKA [6]. Hence, AKAPs form a framework complex that synchronizes multi-protein signaling. In particular, muscle-selective AKAP (mAKAP, AKAP6, AKAP100), a 250-kDa protein, acts as a multi-signaling network protein by recruiting and coordinating cAMP-specific phosphodiesterase (PDE4D3) and compiling a negative feedback loop by assembling PDE4D3 and PKA at the perinuclear membrane [7,8]. PKA phosphorylation of PDE4D3 at Ser13 increases the affinity of PDE4D3 for mAKAP, enhancing its recruitment and allowing for rapid signal termination through PKA phosphorylation of PDE4D3 at Ser54; mAKAP binds to the N-terminal residues of PDE4D3, and PDE4D3 binds to the N-terminal residues 1286–1831 of mAKAP [9].

Besides calcineurin (PP2B), adenylyl cyclase V, and Epac1, mAKAP can also bind protein phosphatase 2A (PP2A), which participates in PDE4D3 dephosphorylation [6,7]. This pattern provides all the required machinery for cAMP synthesis, function, and degradation, which may cause local variation in cAMP and pulsation of localized PKA activity.

Additionally, mAKAP associates with ryanodine receptors (RyR2) on the sarcoplasmic reticulum of myocytes [10,11] as well as the perinuclear endoplasmic reticulum, via three spectrin repeats [6]. RyR2 regulates muscle contraction, which is involved in the release of calcium (Ca^{2+}) from intracellular stores. Altered RyR2 phosphorylation contributes to impaired contractility in heart failure (HF) [7]. Marx *et al.* have proposed that RyR2 is hyperphosphorylated in the failing myocardium at Ser2809, causing this RyR2 channel to have greater Ca^{2+} sensitivity and thus to become functionally “leaky” [11]. Furthermore, it is well established that mAKAP is important for adrenergic-mediated hypertrophy through the calcineurin–NFATc (nuclear factor of activated T cells) pathway that is activated by the increase of Ca^{2+} release from RyR2. This will lead to calcineurin (PP2B) activation that causes NFATc dephosphorylation and translocation into the nucleus, promoting the activity of several pro-hypertrophic gene factors leading to cardiac hypertrophy [12]. Additionally, it has been shown that reduced PDE4D3 levels in the RyR2 complex of failing human hearts, as well as PDE4D3 inactivation in mice, is associated with progressive cardiomyopathy, accelerated HF, and cardiac arrhythmias [13]. However, the complete molecular mechanisms that explain the correlation between this multi-protein signaling complex and heart diseases remain unclear.

In cardiomyocytes, mAKAP functions to regulate cAMP, to modulate substrate phosphorylation, and to dephosphorylate proteins that are involved in cardiac contractility.

Furthermore, these mAKAP-mediated signaling pathways enable it to also function as a gatekeeper for transcription factors. Considering the many crucial roles of mAKAP, aberrant PKA-mAKAP or PDE4D3-mAKAP anchoring may be the cause of some heart diseases [7,9,11]. The goal of this study is to elucidate the structure–function relationship of mAKAP scaffolding protein and its binding partners (PDE4D3 and PKA-RII) using molecularly defined mAKAP mutants (P1400S, S2195F, and L717V), which may potentially lead to defective compartmentalization, co-localization, and binding using an *in vitro* analysis approach. Given the ability of mAKAP to anchor PKA, PDE4D3, PP2A, and RyR2, we propose that mAKAP mutants may alter the hypertrophic signaling by affecting mAKAP protein partner binding, Ca^{2+} release, and the regulation of downstream gene transcription.

Results

Genomic structure of human mAKAP

mAKAP shows a remarkable role in the development of cardiac hypertrophy. Pare *et al.* showed that myocytes expressing mutant mAKAP lacking the PKA binding domain have impaired hypertrophic signaling [14]. A study by Puckelwartz *et al.* also revealed that a human missense mutation, R374H, found within the nesprin-1 alpha domain (a protein responsible for tethering mAKAP to the nuclear membrane) from a patient with dilated cardiomyopathy, required cardiac transplantation [15]. With the established importance of mAKAP in cardiovascular disease, it is now critical to delineate the functional significance of known mAKAP SNPs and screen for additional mAKAP polymorphisms.

The dbSNP database [National Center for Biotechnology Information (NCBI)], University of California Santa Cruz (UCSC), and Ensemble Genome Browser were searched to identify human mAKAP polymorphisms (Table S1). Figure 1a illustrates the genomic structure of the human mAKAP gene (chromosomal location 14q 11.2–14q 13.1 with a coding sequence of 6960 base pairs and protein sequence of 2319 amino acids; NP_004265.3/Gi 21493045). The schematic illustration of the mAKAP protein identifies the different mAKAP binding domains, including spectrin repeats, RyR2, PDE4D3, PKA-RII, and PP2A. Our search of the dbSNP (NCBI), UCSC, and Ensemble Genome Browser databases revealed potentially important human mAKAP polymorphisms found in exon 4 and exon 13 of the gene. These polymorphisms are located within the PDE4D3 binding site and the area flanking the PKA-RII binding domain. After screening numerous non-synonymous polymorphisms, three different mutations of the human mAKAP protein, (1) P1400S (mutation in the PDE4D3 binding site), (2) S2195F (mutation in the PP2A binding site and also flanking the 3'-PKA-RII), and (3) L717V (mutation surrounding the spectrin repeat domain that anchors mAKAP to the perinuclear membrane), were chosen based on their evolutionary trace (ET) rank (where low values mean a greater potential impact) and the mAKAP domain location in which these mutations occur (rank of 31%, 68%, and 40%), respectively (Table S1). The mAKAP-L717V, mAKAP-P1400S, and mAKAP-S2195F were successfully generated and characterized using the human embryonic kidney (HEK) 293 cell line (Fig. 1b and c). Figure 1c shows that when these three mAKAP mutations were independently expressed in HEK293 cells, their protein expression was not significantly different to wild-type expression ($p = 0.834$; $n = 3$; experiment was performed in triplicate), thus indicating that the mutations did not affect expression.

Mutant mAKAP-P1400S reduces binding to PDE4D3, while mAKAP-S2195F and mAKAP-L717V increase binding to PKA-RII α

To determine whether the activation of cAMP altered the propensity of mAKAP for PDE4D3 and PKA-RII binding, we treated transfected HEK293 cells with forskolin in combination with 3-isobutyl-1-methylxanthine (IBMX). Co-immunoprecipitation assay

showed that there is a significant increase in PDE4D3 binding response to mAKAP-wild type (WT; mAKAP-Myc-DDK) after cell stimulation with forskolin plus IBMX; WT(-): 2.854 ± 1.137 and WT(+): 8.499 ± 1.706 ; $p = 0.033$; $n = 4$; experiment was performed in triplicate; (-) and (+) represent without and with stimulation, respectively (Fig. 2). This observation is consistent with the study of Carlisle Michel *et al.* [9] who showed that mAKAP requires anchored PKA to interact strongly with PDE4D3 and also showed that mAKAP preferentially binds with phosphorylated PDE4D3. Interestingly, we found the human mAKAP mutation in which the substitution of proline at position 1400 by serine (mAKAP-P1400S) eliminated this increase in preferential binding; P1400S(-): 3.28 ± 0.845 ; P1400S(+): 4.01 ± 0.4 ; $p = 0.478$; $n = 4$; experiment was performed in triplicate; (-) and (+) represent without and with stimulation, respectively (Fig. 2). Untransfected HEK293 cell lysate and control vector were used as negative controls.

To corroborate these data, we then used surface plasmon resonance (SPR; Biacore 2000) to achieve a real-time quantitative measurement of binding propensity of mAKAP to either the PDE4D3 protein or the PDE4D3 recognition peptide (PDE4D3 N-terminal residues MMHVNNFPFRRHSWICFDVD; levels of peptide phosphorylation were assessed using mass spectrometry and HPLC, with the peptide being in either the unphosphorylated or the phosphorylated state). The referenced response (flow cell – blank) of the mAKAP-wild-type protein showed an increase in SPR binding for the phosphorylated PDE4D3 peptide as compared to the unphosphorylated peptide. In contrast, SPR results demonstrated that this preference for the phosphorylated state was abrogated with the mAKAP-P1400S mutation, as shown by the drop in SPR binding to PDE4D3 relative to mAKAP-wild type and change in relative K_d from $1.5 \mu\text{M}$ wild type to $2.7 \mu\text{M}$ mAKAP-P1400S mutation (Fig. 3g and h). The mutant mAKAP-P1400S SPR binding referenced response, measured from both the unphosphorylated and phosphorylated PDE4D3 peptides, was within the standard error, thus indicating no preference to the phosphorylated PDE4D3 peptide (Fig. 3e).

Immunoprecipitation experiments of the other two mutations, mAKAP-S2195F and mAKAP-L717V, exhibited a similar PDE4D3 binding response, as observed with wild type (Fig. S2; $n = 4$). This was expected since these mutations are located in regions of the mAKAP protein distant from the PDE4D3 binding site. Validating our results using the PDE4D3 peptide, SPR titrations of mAKAP-P1400S, mAKAP-S2195F, and mAKAP-L717V mutants over a chip immobilized with the full-length PDE4D3 protein also showed that mAKAP-P1400S displayed overall lower referenced response [32 ± 3 response unit (RU)] as compared to the other mAKAP mutations (S2195: 43 ± 1.8 RU and L717V: 44.6 ± 8 RU) (Fig. 4; Fig. S3). Untransfected cells were used as a negative control. Our result therefore suggests that signal modulation of the mAKAP/PKA/PDE complex occurs through changes in PDE binding affinities.

PKA is targeted near its substrates by high-affinity binding of the PKA regulatory subunit to AKAPs [16,17]. This targeting mediates PKA phosphorylation by increasing the concentration of PKA catalytic subunits around its substrates [18]. mAKAP recruits the binding and coordinates the activities of PKA-RII to control cardiac contractility upon beta-adrenergic receptor (β -AR) agonist stimulation [14]. Thus, we tested the hypothesis that a mutation in the PKA-RII binding site flanking area (mAKAP-S2195F) would affect mAKAP binding affinity to PKA-RII and thus be associated with altered PKA-dependent signaling and substrate phosphorylation. We found that mAKAP-S2195F had a significant increase in binding to PKA-RII as compared to mAKAP-wild type after HEK293 cell stimulation; WT(-): 0.58 ± 0.13 and WT(+): 1.35 ± 0.3 (Fig. 4, lanes 1 and 2); S2195F(-): 1.44 ± 0.24 and S2195F(+): 4.0 ± 0.9 [Fig. 4, lanes 3 and 4; $p = 0.01$; $n = 4$; experiment was performed in triplicate; (-) and (+) represent without and with stimulation, respectively]. Interestingly, when we performed the same study principle for mAKAP-L717V, this mutation similarly showed an increase in binding propensity to PKA-RII, when probed

with the anti-PKA-RII antibody as compared to the stimulated cells with mAKAP-wild type; L717V(-): 1.29 ± 0.25 ; L717V(+): 2.5 ± 0.35 (Fig. 4, lanes 5 and 6; $p = 0.033$; $n = 4$; experiment was performed in triplicate). These results suggest that both mAKAP-S2195F and mAKAP-L717V exhibit an increase in binding to PKA-RII as compared to WT-mAKAP.

Differential effect of mAKAP mutations on PKA and PDE activities

Based on the changes we observed in binding response of mutant mAKAP to PDE4D3 or PKA-RII, we examined whether these binding changes were translated into alterations in PKA or PDE activities. We measured both basal and maximal cAMP-stimulated PKA activity in HEK293 cells transfected with mAKAP-wild type, mAKAP-P1400S, mAKAP-S2195F, and mAKAP-L717V. Basal PKA activity (Fig. 5a) was significantly increased in mAKAP-S2195F mutant compared to mAKAP-wild type (WT: 7.40 ± 1.28 pmol/mg; P1400S: 7.35 ± 1.27 pmol/mg; S2195F: 13.53 ± 0.996 pmol/mg; and L717V: 6.962 ± 0.693 pmol/mg; $p = 0.0011$; $n = 3$; experiment was performed in triplicate). However, when we co-transfected these cells with PDE4D3-GFP, the PKA activity returned back to the wild-type levels. Similarly, maximal PKA activity, achieved in the presence of $10 \mu\text{M}$ cAMP (Fig. 5b), was significantly increased in the mAKAP-S2195F mutant compared to wild type (WT: 224.5 ± 11.41 pmol/mg; P1400S: 195.4 ± 10.73 pmol/mg; S2195F: 309.7 ± 34.18 pmol/mg; and L717V: 212.6 ± 10.8 pmol/mg; $p = 0.0001$; $n = 3$; experiment was performed in triplicate). Thus, the increase in binding of mAKAP-S2195F to PKA-RII resulted in an increase in PKA activity, while the other two mutations did not affect the PKA activity. The PKA activity measurement is based on the observation that prolonged localization of PKA to mAKAP will increase the exposure time of the PKA catalytic subunit to downstream PKA substrates to subsequently increase substrate phosphorylation.

Since PDE is responsible for termination of PKA signaling, we therefore measured total PDE activity in HEK293 cells transfected with mAKAP-wild type, mAKAP-P1400S, mAKAP-S2195F, and mAKAP-L717V. As shown in Fig. S4, there was no significant difference among all the different mAKAP mutations compared to wild type ($p = 0.523$; $n = 3$; experiment was performed in triplicate). PDE activity was measured based on the production of adenosine monophosphate (product of cAMP degradation) and absorbance at 620 nm. Untransfected HEK293 cell lysates were used as a control for PDE activity measurements. These results suggest that the three mAKAP mutations did not have a statistical effect on overall PDE enzymatic activity possibly because the activity of other PDE isoforms compensates for the influence of incremental changes in PDE4D3 binding.

Mutant mAKAP facilitates calcium release and pro-hypertrophic marker induction

We hypothesized that the mAKAP-S2195F mutant affects PKA activity and increases the efficiency and specificity of PKA to potentiate Ca^{2+} -induced Ca^{2+} release leading to the increased activity of calcineurin (PP2B). Optimizing the established Ca^{2+} measurement protocols by Ruehr et al. [19] for our Nikon microscope, we showed that mAKAP-S2195F significantly increased Ca^{2+} release from internal stores in response to the addition of forskolin in combination with IBMX as compared to mAKAP-wild type or untransfected Chinese hamster ovary (CHO) cells; WT: 377.2 ± 62.1 and S2195F: 877.5 ± 183 ; $p = 0.016$; $n = 4$ (Fig. 6a and b). In contrast, the mAKAP-P1400S and mAKAP-L717V mutants did not show significant differences in Ca^{2+} release from that observed in controls (data not shown). Finally, we investigated the role of these different mutations on downstream signaling pathways that participate in the hypertrophic response. Western blot analysis showed that mAKAP-S2195F and mAKAP-L717V mutants significantly increased both the calcineurin (PP2B) expression ($p = 0.0045$; $n = 4$; experiment was performed in triplicate; Fig. 7a) and the cAMP-response element binding (CREB) phosphorylation ($p = 0.0001$; $n = 4$;

experiment was performed in triplicate; Fig. 7b). Our studies are consistent with the mAKAP signalosome model suggested by Bauman *et al.*, in which the mAKAP multi-protein complex integrates agonist-stimulated β -ARs with cAMP-activated PKA-dependent signaling in order to coordinate the potentiating of calcium-induced calcium release through the sarcoplasmic reticulum's RyR2 channel. This Ca^{2+} release then activates calcineurin (PP2B) to dephosphorylate NFATc transcription factors, resulting in its nuclear translocation, which enhances the transcription of hypertrophic genes [12]. These results suggest that these two AKAP-S2195F and mAKAP-L717V mutations may participate in modulating hypertrophic gene expression compared to wild-type mAKAP.

Discussion

Sympathetic stimulation of β -ARs stimulates adenylyl cyclase activity that increases cAMP production and activates PKA [20]. PKA is a heterotetramer consisting of two catalytic subunits (C) and two regulatory subunits (R) [21]. The specificity of PKA phosphorylation is conferred in part by binding PKA to AKAPs [22]. Thus, this binding recruits PKA to specific intracellular compartments, bringing them in close proximity to its protein substrates, which are essential in mediating β -AR-induced increase in cardiac contractility upon agonist stimulation [23]. HF is a major public health problem and the leading cause of death in the United States [20]. A common phenotype associated with HF is the development of cardiac hypertrophy [14]. Hypertrophy is defined as a physiological increase in heart size in order to compensate the increase in workload. There are a number of therapeutic agents available for HF management; however, there is still a high inter-individual variability in response to drug treatment. This variability is not attributed to clinical, demographic, or environmental reasons, suggesting that genetic factors may play a critical role in this variability [23].

Most of the current available therapeutic agents target G-protein coupled receptors, especially β -ARs. Thus, current research is focusing on targeting downstream signaling of G-protein coupled receptors and designing specific inhibitors to alter these signaling pathways. For example, ARKct is a peptide inhibitor for the G-protein coupled receptor kinase 2 (GRK2), which competes with the endogenous GRK2-G γ transmembrane binding domain that is essential for GRK2 activation upon agonist binding [24,25]. Animal studies have shown that ARKct overexpression enhances basal and agonist-stimulated cardiac function [26]. These studies may open a new avenue for cardiac disease treatment using small-molecule disruptors. Another study showed that replacement of endogenous mAKAP with a mutant form that does not bind PKA-RII results in pro-hypertrophic cardiomyopathy [14]. Based on these observations, mutant mAKAPs that modify PKA signaling would be expected to result in an altered inotropic response.

All AKAPs have amphipathic helix that consists of 14–18 amino acids. This helix contains hydrophobic amino acids that form the hydrophobic ridge and which are responsible for the binding of AKAPs with the PKA regulatory subunit. Generally, AKAPs bind with high affinity to PKA regulatory (PKA-RII) subunits ($K_d = 1\text{--}5\text{ nM}$) [27]. Most AKAPs bind PKA-RII subunits but some AKAPs may have dual specific function (interact with both RII and RI). Inhibition of PKA/AKAP interaction has been shown to alter PKA phosphorylation as well as increase cardiac contractility [28]. The goal of this study was to investigate the role of different human mAKAP coding polymorphisms in PKA and PDE4D3 signaling by investigating the binding propensity of mAKAP to its protein partners and subsequent PKA activity. Inhibition of PKA-dependent activation may be a novel drug target for the treatment of cardiac disease, through designing small molecules to disrupt AKAP complex formation and interfere with compartmentalized cAMP signaling in cardiac myocytes.

For the purpose of studying the human mAKAP structure–function relationship along with its physiological consequences in changing downstream signaling, in vitro pull-down assays, PKA and PDE activities, Ca^{2+} signaling, and SPR experiments were employed. Our data show that mAKAP-P1400S has attenuated binding to PDE4D3. It is located in the PDE4D3 binding domain and its predicted evolutionary importance (low–intermediate) is consistent with the observed modulatory effect on the binding of mAKAP to PDE4D3. Proline has a distinctive cyclic structure that provides conformational rigidity compared to other amino acids, but its substitution to a serine results in a relatively small perturbation, judging from substitution log-odds [29]. Some loss of rigidity may explain a change in binding affinity of mAKAP to PDE4D3 with overall preservation of the other mAKAP functions. Additionally, our immunoprecipitation data show that mAKAP-S2195F promotes a significant increase in binding affinity to PKA-RII . This serine is predicted to have a strong evolutionary importance and its replacement by a large and hydrophobic phenylalanine is likely to impact function. Indeed, this substitution increases mAKAP binding to PKA-RII , possibly due to an increase in the number of hydrophobic amino acids in the hydrophobic ridge. This increase in PKA-RII binding affinity causes a significant increase in PKA activity, which explains the increase in Ca^{2+} release, calcineurin (PP2B) activity, and CREB phosphorylation. The observed increases in these parameters suggest a possible induction of prohypertrophic gene expression. The increase in PKA activity was solely due to an increase in PKA affinity binding to mAKAP-S2195F and not due to a change in total PDE activity. Basal and maximal PKA activity returned back to the normal wild-type level after cotransfecting the cells with PDE4D3. This suggests that PDE4D3 is still capable of normalizing the enhanced PKA activity that is seen with this S2195F mutant mAKAP. Finally, in regard to mAKAP-L717V, although leucine is predicted to be an evolutionary important residue, its substitution to valine is mostly conservative. As can be seen with mAKAP-L717V, this mutation shows an increase in hypertrophic markers without any change in binding to PDE4D3 or changes in PKA activity, as compared to wild type. This implies that the increase in CREB phosphorylation and calcineurin (PP2B) activity is independent of the PKA pathway. This mAKAP-L717V mutation is located in a region flanking the spectrin repeat domain, which is responsible for mAKAP adherence to the perinuclear membrane. The close proximity of the mAKAP-L717V mutation to the nucleus anchoring region of mAKAP provides a future opportunity to study the reason for the observed increases in calcineurin (PP2B) expression levels and increases in CREB phosphorylation.

In conclusion, mAKAP-S2195F and mAKAP-L717V mutants display modified activity and/or binding propensities to PKA-RII while mAKAP-P1400S exhibited reduced association with PDE4D3. Based on these mutations, altered mAKAP binding to PKA may participate in the development of hypertrophy, which is a common phenotype observed in HF. Hence, blockade of PKA-dependent activation through compounds that can modulate mAKAP complex formation and stability may provide a novel drug target for the treatment of pathological hypertrophy associated with HF. The next step will be to study these mAKAP mutations in animal models of hypertrophy, in order to gain greater insight about these mutations *in vivo* and evaluate differences in drug responses.

Materials and Methods

Computational genomic study

The dbSNP database (NCBI), UCSC, and Ensemble Genome Browser were searched to identify known mAKAP polymorphisms. The evolutionary and functional importance of each residue of the mAKAP protein was estimated by an ET analysis [30], using 24 homologs with divergences from hominid up to fish. These ET ranks represent the fraction

of sequence positions with equal or higher evolutionary impact on divergences among mAKAP homologs such that smaller values translate to higher impact.

Plasmids, transfection, and sample preparation

HEK293 cells were transiently co-transfected with cDNA encoding human PDE4D3-GFP, human RII -GFP, and Myc-DDK-tagged human mAKAP (WT; mAKAP-wild type, mAKAP-P1400S, mAKAP-S2195F, or mAKAP-L717V) (Origene) present in PCMV6 mammalian expression vector using Lipofectamine 2000 reagent (Invitrogen). The transient transfections were performed on 85% confluent cells using plasmid DNAs. The level of HEK293 cell transfections was assessed using immunohistochemistry where the level of transfection was ~70%. After 48 h of transfection, cells were serum starved for 5 h and then stimulated with 10 μ M forskolin and 50 μ M IBMX or dimethyl sulfoxide control for 15 min. Cells were lysed with lysis buffer [10 mM Na₂PO₄, pH 7.4, 150 mM NaCl, 5 mM EDTA (ethylenediaminetetraacetic acid), 5 mM EGTA [ethylene glycol bis(-aminoethyl ether) *N,N*-tetraacetic acid], 1% (v/v) Triton X-100, 1 mM benzamidine, and protease inhibitor cocktail (Sigma)], and extracts were cleared by centrifugation at 16,000g for 10 min.

Immunoprecipitation

Equal amounts of protein were immunoprecipitated with anti-FLAG M2-affinity gel (Sigma). For the negative control, 1 mL of lysis buffer [10 mM Na₂PO₄, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% (v/v) Triton X-100, 1 mM benzamidine, and protease inhibitor cocktail (Sigma)] with no FLAG-protein, untransfected cell lysate, and cell lysate containing control vector were used. The resin was washed three times with 0.5 mL of TBS (50 mM Tris-HCl and 150 mM NaCl, pH 7.4); bound proteins were eluted using SDS-PAGE sample buffer, resolved by SDS/PAGE (3–8% Tris-acetate gel), and transferred onto nitrocellulose membrane. Bound proteins were detected with anti-mAKAP (1:1000, Millipore), anti-RII (1:100, Santa Cruz), anti-PDE4D3 (1:500, Abcam), and anti-PP2A (1:500, Millipore) antibodies. For densitometry, bands were quantified using FluorChem 8800 software. The amount of PDE4D3, PKA-RII, or PP2A bound was normalized to the amount of mAKAP immunoprecipitated.

Surface plasmon resonance

SPR experiments were performed to directly measure and confirm the binding of mAKAP with PDE4D3 using a Biacore 2000. The CM5 chip was primed three times with phosphate-buffered saline running buffer and washed for 1 min with 50 mM NaOH at 10 μ L/min to precondition the surface. A sample of PDE4D3-purified protein (100 μ g/mL stock) was prepared to 25 μ g/mL in 160 μ L of immobilization buffer (10 mM sodium acetate, pH 4.14) and immobilized on flow cell 2. A second chip was prepared with both PDE peptide (MMHVNNFPFRRHSWICFDVD) on flow cell 2 and a phosphorylated peptide at position 13 [MMHVNNFPFRRHS(p)WICFDVD] (GenScript) on flow cell 4. The synthesis of the phosphorylated peptide was validated via mass spectrometry and HPLC by GenScript. The mAKAP and mAKAP mutant (HEK293 transfected cell lysate) samples were injected over a range of concentrations. Experiments were run in triplicate and as twofold serial dilutions to explore the interactions. The referenced response (RU) was calculated by subtracting the blank flow cell 1 or 3 from the signal measured from the immobilized flow cell 2 or 4 to remove baseline effects, and the delta response between the phosphorylated and unphosphorylated peptide was calculated by subtracting flow cell 2 from 4 and analyzed using Scrubber2 (Version 2.0 Center for Biomolecular Interactions Analysis, University of Utah) and Matlab. Cell lysate without mAKAP overexpression was used as a negative control.

PKA activity

PKA activity was determined via an enzyme immunoassay kit (Enzo Life Sciences) according to the manufacturer's instructions. Stimulated PKA activation was performed by incubating with 10 μ M cAMP (Sigma) for 10 min at 37 °C. The reaction was stopped by the addition of kinase assay dilution buffer. PKA activity was then measured according to manufacturer's instructions. The PKA activity assay was done in linear activity range. Each experiment was performed in triplicate. Relative PKA activity was normalized to milligrams of protein.

PDE activity

PDE activity was measured using a cyclic nucleotide PDE colorimetric assay kit (Enzo Life Sciences) according to the manufacturer's instructions. Samples were desalted by column chromatography to remove excess phosphate and nucleotides and tested for effective removal of phosphate/nucleotides from the extract by adding 1 μ L of extract to 100 μ L of BIOMOL Green. Assay was performed by the addition of 0.5 mM 3',5'-cAMP, 15 μ L assay buffer, and 10 μ L 5'-nucleotidase to the samples and then the plate was incubated for 45 min at 30 °C. The reaction was terminated by the addition of 100 μ L of BIOMOL Green reagent, and the absorbance was measured at OD₆₂₀ (optical density at 620 nm) in a microplate-reading spectrophotometer. The PDE activity assay was done in linear activity range. Each experiment was performed in triplicate.

Calcium signaling measurement assay

CHO cells prior to passage 7 were stably co-transfected with human mAKAP (wild type or mutant) and PDE4D3-GFP plasmid DNA using Lipofectamine 2000 transfecting reagent (Invitrogen). G418 (Invitrogen) was used for selection, based on the manufacturer's instructions. Then, transfected cells were cultured in 12-well glass-bottom plates with F-12K medium containing 1% antibiotics overnight. Plated cells were incubated with Fluo8-AM dye (excitation, 490 nm; emission, 525 nm), dissolved in F-12K medium containing 1% antibiotics supplemented with 2.5 mM probenecid acid and 0.2% pluronic F-127 for 15 min. The cells were washed with washing buffer three times (F-12K medium containing 1% antibiotics supplemented with 2.5 mM probenecid acid and 1% pluronic F-68) [31]. After 10 s recording as baseline, each well of cells was individually stimulated with 10 μ M forskolin and 50 μ M IBMX for calcium (Ca^{2+}) signal in a reaction volume of 1 mL washing buffer using the Nikon Ti-S eclipse microscope (40 $\times$ objective) for 1 min recording.

Immunoblotting

Immunoblot analysis was performed as previously described using antibodies for GAPDH (cell signaling), mAKAP (Millipore), PDE4D3 (Abcam), PKA-RII (Santa Cruz), Myc-DDk (Origene), total and phospho-CREB (Millipore), PP2B-A (Santa Cruz), and PP2A (Millipore). Cell lysate samples were resolved by SDS-PAGE gradient and then transferred to polyvinylidene difluoride membranes. Blots were then incubated overnight at 4 °C with primary antibodies. The blots were washed three times with TBS containing 0.1% Tween-20 (TBST) and then probed with secondary antibodies (Cell Signaling). Blots were developed by an enhanced chemiluminescence kit (Pierce). The bands were quantified using the FluorChem 8800 software.

Statistical analysis

Data are represented as mean $\pm$ SEM. Data were analyzed with one-way ANOVA followed by Tukey–Kramer's post hoc test or by Student's *t* test where appropriate. Statistical significance was determined by a *p* value < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Robert Schwartz (Department of Biology and Biochemistry, University of Houston) for the use of the Biacore 2000 instrument. This work was supported by National Institutes of Health grants HL085487 (to B.K.M.) and GM79656 and GM66099 (to O.L.) and by the National Science Foundation through NSF CCF-0905536 and DBI-1062455 (to O.L.).

Abbreviations used

AKAP	A-kinase anchoring protein
mAKAP	muscle-selective A-kinase anchoring protein
PDE4D3	phosphodiesterase-4D3
PDE	phosphodiesterase
RyR2	ryanodine receptors
PP2A	protein phosphatase 2A
PP2B	protein phosphatase 2B, calcineurin
CREB	cAMP-response element binding
-AR	beta-adrenergic receptor
PKA	protein kinase A
WT	wild type
SPR	surface plasmon resonance
RU	response unit
GRK2	G-protein coupled receptor kinase 2
NFATc	nuclear factor of activated T cells
CHO	Chinese hamster ovary
HEK	human embryonic kidney
IBMX	3-isobutyl-1-methylxanthine
NCBI	National Center for Biotechnology Information
UCSC	University of California Santa Cruz
HF	heart failure
ET	evolutionary trace

References

1. Walsh DA, Van Patten SM. Multiple pathway signal transduction by the cAMP-dependent protein kinase. *FASEB J.* 1994; 8:1227–1236. [PubMed: 8001734]
2. Colledge M, Scott JD. AKAPs: from structure to function. *Trends Cell Biol.* 1999; 9:216–221. [PubMed: 10354567]
3. Dell'Acqua ML, Scott JD. Protein kinase A anchoring. *J Biol Chem.* 1997; 272:12881–12894. [PubMed: 9190362]

4. Christian F, Szaszak M, Friedl S, Drewianka S, Lorenz D, Goncalves A, et al. Small molecule AKAP-protein kinase A (PKA) interaction disruptors that activate PKA interfere with compartmentalized cAMP signaling in cardiac myocytes. *J Biol Chem.* 2011; 286:9079–9096. [PubMed: 21177871]
5. Dodge-Kafka KL, Soughayer J, Pare GC, Carlisle Michel JJ, Langeberg LK, Kapiloff MS, et al. The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. *Nature.* 2005; 437:574–588. [PubMed: 16177794]
6. Kapiloff MS, Jackson N, Airhart N. mAKAP and the ryanodine receptor are part of a multi-component signaling complex on the cardiomyocyte nuclear envelope. *J Cell Sci.* 2001; 114:3167–3176. [PubMed: 11590243]
7. Kritzer MD, Li J, Dodge-Kafka K, Kapiloff MS. AKAPs: the architectural underpinnings of local cAMP signaling. *J Mol Cell Cardiol.* 2012; 52:351–368. [PubMed: 21600214]
8. Dodge KL, Khouangsathien S, Kapiloff MS, Mouton R, Hill EV, Houslay MD, et al. mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. *EMBO J.* 2001; 20:1921–1930. [PubMed: 11296225]
9. Carlisle, Michel JJ.; Dodge, KL.; Wong, W.; Mayer, NC.; Langeberg, LK.; Scott, JD. PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex. *Biochem J.* 2004; 381:587–592. [PubMed: 15182229]
10. Yang J, Drazba JA, Ferguson DG, Bond M. A-kinase anchoring protein 100 (AKAP100) is localized in multiple subcellular compartments in the adult rat heart. *J Cell Biol.* 1998; 142:511–522. [PubMed: 9679148]
11. Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblyt N, et al. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell.* 2000; 101:365–376. [PubMed: 10830164]
12. Bauman AL, Michel JJ, Henson E, Dodge-Kafka KL, Kapiloff MS. The mAKAP signalosome and cardiac myocyte hypertrophy. *IUBMB Life.* 2007; 59:163–179. [PubMed: 17487687]
13. Lehnart SE, Wehrens XH, Reiken S, Warrier S, Belevych AE, Harvey RD, et al. Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. *Cell.* 2005; 123:25–35. [PubMed: 16213210]
14. Pare GC, Bauman AL, McHenry M, Michel JJ, Dodge-Kafka KL, Kapiloff MS. The mAKAP complex participates in the induction of cardiac myocyte hypertrophy by adrenergic receptor signaling. *J Cell Sci.* 2005; 118:5637–5646. [PubMed: 16306226]
15. Puckelwartz MJ, Kessler EJ, Kim G, Dewitt MM, Zhang Y, Earley JU, et al. Nesprin-1 mutations in human and murine cardiomyopathy. *J Mol Cell Cardiol.* 2010; 48:600–608. [PubMed: 19944109]
16. Zakhary DR, Fink MA, Ruehr ML, Bond M. Selectivity and regulation of A-kinase anchoring proteins in the heart. The role of autophosphorylation of the type II regulatory subunit of cAMP-dependent protein kinase. *J Biol Chem.* 2000; 275:41389–41395. [PubMed: 10993882]
17. Carr DW, Stofko-Hahn RE, Fraser ID, Bishop SM, Acott TS, Brennan RG, et al. Interaction of the regulatory subunit (RII) of cAMP-dependent protein kinase with RII-anchoring proteins occurs through an amphipathic helix binding motif. *J Biol Chem.* 1991; 266:14188–14192. [PubMed: 1860836]
18. Fink MA, Zakhary DR, Mackey JA, Desnoyer RW, Apperson-Hansen C, Damron DS, et al. AKAP-mediated targeting of protein kinase A regulates contractility in cardiac myocytes. *Circ Res.* 2001; 88:291–397. [PubMed: 11179196]
19. Ruehr ML, Russell MA, Ferguson DG, Bhat M, Ma J, Damron DS, et al. Targeting of protein kinase A by muscle A kinase-anchoring protein (mAKAP) regulates phosphorylation and function of the skeletal muscle ryanodine receptor. *J Biol Chem.* 2003; 278:24831–24846. [PubMed: 12709444]
20. Bers DM. Cardiac excitation-contraction coupling. *Nature.* 2002; 415:198–205. [PubMed: 11805843]
21. Scott JD. Cyclic nucleotide-dependent protein kinases. *Pharmacol Ther.* 1991; 50:123–135. [PubMed: 1653962]

22. Wehrens XH, Lehnart SE, Reiken S, Vest JA, Wronska A, Marks AR. Ryanodine receptor/calcium release channel PKA phosphorylation: a critical mediator of heart failure progression. *Proc Natl Acad Sci USA*. 2006; 103:511–528. [PubMed: 16407108]
23. Rubin CS. A kinase anchor proteins and the intracellular targeting of signals carried by cyclic AMP. *Biochim Biophys Acta*. 1994; 1224:467–479. [PubMed: 7803506]
24. Rockman HA, Chien KR, Choi DJ, Iaccarino G, Hunter JJ, Ross J Jr, et al. Expression of a beta-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. *Proc Natl Acad Sci USA*. 1998; 95:7000–70015. [PubMed: 9618528]
25. Harding VB, Jones LR, Lefkowitz RJ, Koch WJ, Rockman HA. Cardiac beta ARK1 inhibition prolongs survival and augments beta blocker therapy in a mouse model of severe heart failure. *Proc Natl Acad Sci USA*. 2001; 98:5809–5814. [PubMed: 11331748]
26. Keys JR, Koch WJ. The adrenergic pathway and heart failure. *Recent Prog Horm Res*. 2004; 59:13–30. [PubMed: 14749495]
27. Kinderman FS, Kim C, von Daake S, Ma Y, Pham BQ, Spraggon G, et al. A dynamic mechanism for AKAP binding to RII isoforms of cAMP-dependent protein kinase. *Mol Cell*. 2006; 24:397–408. [PubMed: 17081990]
28. McConnell BK, Popovic Z, Mal N, Lee K, Bautista J, Forudi F, et al. Disruption of protein kinase A interaction with A-kinase-anchoring proteins in the heart in vivo: effects on cardiac contractility, protein kinase A phosphorylation, and troponin I proteolysis. *J Biol Chem*. 2009; 284:1583–1592. [PubMed: 18945669]
29. Henikoff S, Henikoff JG. Amino acid substitution matrices from protein blocks. *Proc Natl Acad Sci USA*. 1992; 89:10915–10929. [PubMed: 1438297]
30. Lichtarge O, Bourne HR, Cohen FE. An evolutionary trace method defines binding surfaces common to protein families. *J Mol Biol*. 1996; 257:342–358. [PubMed: 8609628]
31. Chillar AJ, Karimi P, Tang K, Ruan KH. An agonist sensitive, quick and simple cell-based signaling assay for determination of ligands mimicking prostaglandin E2 or E1 activity through subtype EP1 receptor: suitable for high throughput screening. *BMC Complement Altern Med*. 2011; 11:11. [PubMed: 21299883]

Fig. 1.

Human mAKAP genomic structure. (a) Human mAKAP genomic structure illustrating different mAKAP binding domains. (b) Characteristics of the three different human mAKAP mutations defined in this study, showing the amino acid change and their binding position in mAKAP. (c) Representative immunoblot depicting protein expression of wild-type mAKAP (lane 1), mAKAP-P1400S (lane 2), mAKAP-S2195F (lane 3), and mAKAP-L717V (lane 4). No significant effect of these point mutations in regard to protein expression was observed ($p = 0.834$). Data are expressed as the mean $\pm$ SEM (performed in three independent experiments).

(a) IP:Flag myc-DDK

mAKAP-Wt	-	+	+	+	+	-	-	-	-
mAKAP-P1400S	-	-	-	-	-	+	+	+	+
PDE4D3-GFP	-	-	-	+	+	-	-	+	+
Forskolin/IBMX	-	+	-	+	-	+	-	+	-
Control	+	-	-	-	-	-	-	-	-

IP: Flag myc-DDK

(b) **Fig. 2.**

Mutant mAKAP binding to PDE4D3. Representative immunoblot depicting (a) precipitated myc-DDK tag mAKAP-wild type (lanes 2–5) and mAKAP-P1400S (lanes 6–9), with (lanes 4, 5, 8, and 9) and without (lanes 2, 3, 6, and 7) PDE4D3-GFP. The blot also represents the untransfected HEK293 cell lysate as a control (lane 1). Anti-Flag myc-DDK affinity chromatography columns (Sigma) were used to immunoprecipitate mAKAP.

Immunoprecipitates were eluted using $2 \times$ SDS sample buffer and then probed with anti-mAKAP (Millipore) and antiPDE4D3 (Abcam) antibodies. (b) The bar graph displays the relative binding based on densitometric quantification of the immunoblot bands using Alpha Innotech Fluorchem 8000 software. The upper panel represents a Western blot probed with

anti-PDE4D3 while the lower panel represents a Western blot probed with anti-mAKAP antibody. Wild-type mAKAP (lane 2) shows a significant increase in binding propensity to PDE4D3 compared to mAKAP-P1400S (lane 4) upon cell stimulation. The amount of PDE4D3 bound to mAKAP was quantified and normalized to the amount of mAKAP immunoprecipitated. The immunoblots and columns are labeled with transfection and stimulation conditions (where – represents no cell stimulation and + represents cell stimulation with 10 μ M forskolin and 50 μ M of IBMX). Data are expressed as the mean $\pm$ SEM (performed in four independent experiments); * $p < 0.05$.

Fig. 3.

SPR delta RUs of mAKAP binding to PDE4D3. SPR referenced RUs of (a) mAKAP-wild type (WT)/PDE4D3 (PDE) phosphorylated peptide (blue trace) and mAKAP-P1400S (P1400S) mutation/PDE4D3 (PDE) phosphorylated peptide (black trace), summarized in the bar graph (b), show that mAKAP-P1400S significantly reduces binding to PDE4D3, as compared to wild-type mAKAP. The SPR referenced RU of (c) mAKAP-wild type/PDE4D3 phosphorylated peptide (blue trace) and mAKAP-wild type/PDE4D3 unphosphorylated peptide (red trace), summarized in the bar graph (d), shows that phosphorylated PDE4D3 peptide significantly increases binding to mAKAP-wild type, as compared to unphosphorylated PDE4D3 peptide. The SPR referenced RU of (e) mAKAP-P1400S/

PDE4D3 phosphorylated peptide (black trace) and mAKAP-P1400S/PDE4D3 unphosphorylated peptide (magenta trace), summarized in the bar graph (f), shows that mAKAP-P1400S reduces the preferential response to the phosphorylated PDE4D3 peptide. (g) Serial 2× dilution of wild-type mAKAP (continuous line) and the model fit (broken line) resulted in a relative K_d of 1.5 μM , while (h) serial 2× dilution of mAKAP-P1400S (continuous line) and its model fit (broken line) resulted in a relative K_d of 2.7 μM . For (a) to (f), the referenced RU is the blank subtracted from the flow cell [flow cell (Fc) – blank]. For (g) and (h), the delta response is the phosphorylated cell subtracted from the unphosphorylated flow cell for each injection [flow cell (Fc4) – (Fc2)]. Both the unphosphorylated and phosphorylated (S^{13}) PDE4D3 peptides were immobilized on the same CM5 chip. Wild-type mAKAP and mAKAP mutant samples were injected over a range of concentrations as twofold serial dilutions run in triplicate to explore the pattern of the interaction and analyzed using Scrubber2 (Version 2.0 Center for Biomolecular Interactions Analysis, University of Utah) and Matlab with a 1:1 Langmuir model. Cell lysate as well as reference flow cell were used as a negative control. Data are expressed as the mean $\pm$ SEM; * $p < 0.05$.

IP: Flag myc-DDK

Fig. 4. mAKAP-S2195F and mAKAP-L717V mutant binding propensity to PKA-RII. The results show precipitated myc-DDK tag mAKAP-wild type (lanes 1 and 2), mAKAP-S2195F (lanes 3 and 4), and mAKAP-L717V (lanes 5 and 6), where RII-GFP was pulled down using the anti-myc-DDK antibody (anti-Flag affinity chromatography). Immunoprecipitates were probed with anti-mAKAP (Millipore) to detect mAKAP (lower panels) and anti-RII (Santa Cruz) antibody to detect the precipitated PKA-RII (upper panels). The bar graph displays the relative binding based on densitometric quantification of the immunoblot bands using Alpha Innotech Fluorchem 8000 software. Both mAKAP mutants, S2195F and L717V, show a significant increase in binding propensity to PKA-RII, as compared to wild-type mAKAP after cell stimulation. The amount of PKA-RII bound to mAKAP was quantified and normalized to the amount of mAKAP immunoprecipitated. The immunoblots and columns are labeled with transfection and stimulation conditions (where - represents no cell stimulation and + represents cell stimulation with 10 μ M forskolin and 50 μ M IBMX). Data are expressed as the mean $\pm$ SEM (performed in four independent experiments); * p < 0.05.

Fig. 5. Basal and stimulated PKA activity in wild-type and mutant mAKAP cell lysates. Basal (a) and maximal (b) PKA activity in wild type and mutant (P1400S, S2195F, and L717V) mAKAP-transfected HEK293 cell lysates, in the absence or presence of PDE4D3 (PDE). Maximal PKA activity was determined in the presence of 10 μ M cAMP. PKA activity was normalized to picomoles per milligram. The mAKAP-S2195F showed a significant increase in PKA activity under both basal and stimulated conditions. Data are expressed as the mean $\pm$ SEM (performed in triplicate); * $p < 0.05$.

Fig. 6. Calcium transient and peak fluorescence intensity of wild-type and mutant mAKAP-S2195F-transfected CHO cells. (a) Calcium transient fluorescence intensity signal of mAKAP-wild type (black squares) and mAKAP-S2195F (black triangles) in stably transfected CHO cells. CHO cells were grown on glass-bottom plates and incubated with Fluo8-AM. CHO cells were stimulated by the addition of 10 μ M forskolin and 50 μ M IBMX (arrow). Calcium signals were obtained using a Nikon Ti-S eclipse microscope and plotted as fluorescence intensity. As a control, the fluorescence intensity of untransfected CHO cells was also measured (black circles). (b) The bar graph represents the peak calcium fluorescence intensity from stably transfected CHO cells with mAKAP-wild type and

mAKAP-S2195F mutant, following stimulation with 10 μ M forskolin and 50 μ M IBMX. Data are normalized to control (CHO cells transfected with control plasmid) and expressed as the mean $\pm$ SEM (performed in triplicate); * $p < 0.05$.

Fig. 7.

PKA-dependent substrate phosphorylation in wild-type and mutant mAKAP in cell lysates. Western blot analysis of PKA-dependent substrate phosphorylation of (a) calcineurin (PP2B) and (b) phospho-CREB from HEK293 cells expressing wild-type mAKAP or mutant mAKAP (P1400S, S2195F, and L717V) and containing PDE4D3. HEK293 cells were stimulated with 10 μ M forskolin and 50 μ M IBMX. For each substrate, the upper panel shows a Western blot with the anti-protein antibody and the lower panel shows a Western blot with an antibody to GAPDH or total CREB (lane 1, mAKAP-wild type; lane 2, mAKAP-P1400S; lane 3, mAKAP-S2195F; lane 4, mAKAP-L717V). The bar graphs show

the ratio of the protein expression normalized to GAPDH or t-CREB. Data are expressed as the mean $\pm$ SEM; n = 4; * p < 0.05.