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Targeting of Protein Kinase A by Muscle A Kinase-anchoring Protein (mAKAP) Regulates Phosphorylation and Function of the Skeletal Muscle Ryanodine Receptor*

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Protein kinase A anchoring proteins (AKAPs) tether cAMP-dependent protein kinase (PKA) to specific subcellular locations. The muscle AKAP, mAKAP, co-localizes with the sarcoplasmic reticulum Ca^{2+} release channel or ryanodine receptor (RyR). The purpose of this study was to determine whether anchoring of PKA by mAKAP regulates RyR function. Either mAKAP or mAKAP-P, which is unable to anchor PKA, was expressed in CHO cells stably expressing the skeletal muscle isoform of RyR (CHO-RyR1). Immunoelectron microscopy showed that mAKAP co-localized with RyR1 in disrupted skeletal muscle. Following the addition of 10 μM forskolin to activate adenylyl cyclase, RyR1 phosphorylation in CHO-RyR1 cells expressing mAKAP increased by 42.4 ± 6.6% (n = 4) compared with cells expressing mAKAP-P. Forskolin treatment alone did not increase the amplitude of the cytosolic Ca^{2+} transient in CHO-RyR1 cells expressing mAKAP or mAKAP-P; however, forskolin plus 10 mM caffeine elicited a cytosolic Ca^{2+} transient, the amplitude of which increased by 22% ($p < 0.05$) in RyR1/mAKAP-expressing cells compared with RyR1/mAKAP-P-expressing cells. Therefore, localization of PKA by mAKAP at RyR1 increases both PKA-dependent RyR phosphorylation as well as efflux of Ca^{2+} through the RyR. Therefore, RyR1 function is regulated by mAKAP targeting of PKA, implying an important functional role for PKA phosphorylation of RyR in skeletal muscle.

Cyclic AMP-dependent protein kinase (PKA)[1] has a wide range of substrates and elicits a variety of cellular responses. Protein kinase A anchoring proteins (AKAPs) modulate PKA-dependent phosphorylation by tethering this kinase to a specific subcellular location to regulate an otherwise ubiquitous signal and allow specific intracellular changes to occur. In some cases, these substrates may be bound to or are located near an AKAP. For example, the AKAP yotiao binds to its substrate, the *N*-methyl-D-aspartate receptor. Co-expression of these two proteins increases PKA-dependent potentiation of channel currents (1). Another AKAP, AKAP79, co-immunoprecipitates with the β_2-adrenergic receptor (β_2-AR) together with PKA, protein kinase C, and protein phosphatase 2B (calcineurin) (2). In HEK293 cells expressing AKAP79 and β_2-AR, phosphorylation of the β_2-AR was decreased when the interaction between AKAP79 and the receptor was disrupted (2).

In other cases, AKAP anchoring of PKA can influence the phosphorylation state of substrates not in close proximity to the AKAP. For example, forskolin stimulation of HEK293 cells expressing AKAP75, which targets PKA to the cytoskeleton, resulted in a 5–10-fold higher phosphorylation of cAMP response element-binding protein compared with control cells (3). We previously demonstrated that upon isoproterenol stimulation, PKA-dependent phosphorylation of two myofibrillar proteins, troponin I and myosin-binding protein C, was significantly reduced in cardiac myocytes expressing Ht31 (4), a peptide that binds the regulatory subunit (RII) of type II PKA and prevents AKAP-PKA interactions (5). To date, no AKAP has been found to co-localize with the myofibrils in cardiac myocytes. Thus, the phosphorylation state of these two substrates may be regulated by an AKAP not in close proximity to troponin I or myosin-binding protein C. In some cases AKAPs may anchor PKA within a region of the cell subject to local elevated levels of cAMP (6). Thus, upon the appropriate stimulus, local transient increases in cAMP concentration could activate anchored PKA (7).

Other groups have reported changes in cell function when the interaction between AKAP and PKA is disrupted. For example, PKA anchoring was required in order to maintain AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid) responsive glutamate receptor currents in cultured hippocampal neurons (8). Both insulin secretion and increased intracellular Ca^{2+} are inhibited in pancreatic islet cells following treatment with Ht31, and cell-permeant forms of Ht31 inhibit PKA-dependent sperm motility (9, 10). In cardiac myocytes, Ht31 reduces isoproterenol-dependent potentiation of Ca^{2+} current through the L-type Ca^{2+} channel. Disrup-

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[1] The abbreviations used are: PKA, protein kinase A; AKAP, protein kinase A anchoring proteins; mAKAP, muscle AKAP; β_2-AR, β_2-adrenergic receptor; jSR, junctional sarcoplasmic reticulum; DHPR, dihydropyridine receptor (L-type voltage sensitive Ca^{2+} receptor); RyR1, ryanodine receptor type 1; PDE4D3, phosphodiesterase 4D3; AKAP15/18, A kinase-anchoring protein 15/18; RII, PKA regulatory subunit type II; C, PKA catalytic subunit; CHO, Chinese hamster ovary; TBS, Tris-buffered saline; ER, endoplasmic reticulum; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein.

tion of the interaction between AKAP79 and the β_2-AR attenuates downstream activation of the mitogen-activated protein kinase pathway (2, 11).

In both cardiac and skeletal muscle, mAKAP (formerly AKAP100) is highly expressed. In cardiac muscle, this AKAP has been immunolocalized to the perinuclear membrane and to the junctional sarcoplasmic reticulum (jSR) (12–14). In both skeletal and cardiac muscle, there are several PKA substrates in this highly specialized region of the jSR/transverse tubule, notably the dihydropyridine receptor (DHPR) or L-type voltage-sensitive Ca^{2+} channel and the ryanodine-sensitive Ca^{2+} release channel (RyR). Jones *et al.* (15) found no significant PKA II activity associated with highly purified cardiac junctional SR membrane preparation, whereas Marx *et al.* (16) showed that in cardiac muscle mAKAP co-immunoprecipitates with the cardiac-specific isoform of the RyR (RyR2). These investigators also recently showed that PKA and mAKAP co-immunoprecipitated with the skeletal-specific type I isoform of the RyR (RyR1) (17).

It is well established that PKA can regulate RyR2 function. Activation of β-adrenergic receptors results in a time-dependent increase in RyR2 phosphorylation (18). PKA-dependent phosphorylation of single RyR2 channels increases mean open probability, open frequency, and open time (19). In pancreatic β-cells, the activation of PKA by forskolin increases the caffeine-dependent Ca^{2+} transient through RyR2 (20). In general, PKA-dependent phosphorylation of RyR2 increases Ca^{2+} efflux from the SR into the cytosol. However, little information is currently available on the effect of PKA phosphorylation of RyR1. Phosphorylation of the channel can regulate its function (21, 22). This is further supported by recent evidence that calcineurin (protein phosphatase 2B) co-localizes with RyR1 in a mouse skeletal muscle cell line, implying a role for dephosphorylation of this channel (23). Calcineurin inhibition by cyclosporin also enhances caffeine-induced Ca^{2+} release, thus implying a functional role for RyR1 phosphorylation (23). Although mAKAP co-immunoprecipitates with RyR2 (16) and RyR1 (17), a direct functional effect of mAKAP targeting of PKA at the RyR on RyR function has not been observed. Using CHO cells stably expressing RyR1 (24), we have addressed the question of whether mAKAP anchoring of PKA facilitates PKA-dependent RyR phosphorylation and, in doing so, alters RyR function.

EXPERIMENTAL PROCEDURES

Construction of Vectors for the Expression of ECFP-tagged mAKAP or mAKAP-P—Cloning of mAKAP is described by Kapiloff *et al.* (14). Full-length mAKAP or a mutant form of mAKAP, mAKAP-P (I2062P), which does not bind PKA, was ligated into pEGFP to obtain green fluorescent fusion proteins (14). The substitution of a proline for an isoleucine disrupts the amphipathic helix of the RII-binding domain of mAKAP (I2062P), thus abolishing RII binding. The cDNA coding for EGFP was excised from mAKAP-EGFP or mAKAP-P-EGFP using *Age*I and *Bsr*GI and replaced with cDNA encoding either ECFP (a gift from Roger Tsien, Howard Hughes Medical Institute, University of California, San Diego, CA) to create mAKAP-ECFP or mAKAP-P- ECFP, respectively.

Transfection and Selection of Positive Cells—CHO cells stably expressing RyR1-EGFP (24–26) were plated on 30-mm glass bottom dishes and transiently transfected with either mAKAP-ECFP or mAKAP-P-ECFP using LipofectAMINE Plus (Invitrogen) according to the manufacturer's protocol. This cell line is routinely used as a model system for investigating RyR function (24–27). We initially planned to use the ECFP tag to sort mAKAP-ECFP or mAKAP-P-ECFP-positive cells and to create stable cell lines expressing either of these fusion proteins. However, after seven passages, these cells showed a diminished Ca^{2+} response in the presence of caffeine. Therefore, we routinely transfected RyR1-EGFP cells with mAKAP-ECFP or mAKAP-P-ECFP between passages 3 and 7, and these cells were used 48 h after transfection. For brevity, mAKAP-ECFP, mAKAP-P-ECFP, or RyR1-EGFP will be referred to as mAKAP, mAKAP-P, or RyR1, respectively. The addition of a green fluorescent protein tag does not affect the function or targeting of RyR1 (24, 28) or mAKAP (14, 29).

Subcellular Localization of mAKAP by Immunoelectron Microscopy—The rectus femoris muscle from an adult rat was gently disrupted with a single passage through a Dounce apparatus. All of the solution changes were performed by sedimentation of the tissue in a clinical Centrifuge followed by resuspension of the loose pellet in the appropriate solution. Samples were washed three times for 20 min each in phosphate-buffered saline containing 100 mM sucrose and 10 mg/ml bovine serum albumin. Samples were then incubated overnight with mAKAP antibody (1:10 dilution, Upstate Biotechnology). The unbound primary antibody was removed with three 20-min washes as above, and the samples were then incubated overnight with goat anti-rabbit IgG immunogold secondary antibodies (1:10, Jackson ImmunoResearch, West Grove, PA). Control samples were left in wash solution during the 12-h period that experimental samples were exposed to primary antibody and were then incubated with secondary immunogold in parallel with the experimental samples. To remove unbound immunogold, tissues were washed twice with phosphate-buffered saline and once with 10 mM Na^+-cacodylate buffer, pH 7.2. Tissues were prepared for electron microscopy using routine fixation, dehydration, and embedding protocols. Thin sections were stained with lead citrate and uranyl acetate and examined in a JEOL 100CX transmission electron microscope.

Western Blot Analysis and Measurement of RyR Phosphorylation in CHO Cells—Western blot analysis was used to verify that transfected CHO cells expressed the appropriate fusion proteins and also to investigate whether the phosphorylation state of RyR is altered in the presence or absence of mAKAP or mAKAP-P following the activation of PKA. Cells were grown to 95% confluency in a T-75 culture flask. PKA activity in the transfected CHO cells was stimulated by exposing cells for 5 min to 10 μM forskolin, an activator of adenylyl cyclase. Adherent cells were released by incubating with ice-cold M-PER mammalian protein extraction buffer (Pierce) containing a protease and phosphatase inhibitor mixture (Sigma). Proteins from the cell lysate were separated using SDS-PAGE (5% acrylamide gel), transferred to nitrocellulose membrane, and probed with mouse anti-RyR (1:1000, Affinity Bioreagents), rabbit anti-mAKAP (1:1000, polyclonal, Upstate Biotechnology), or rabbit anti-phosphoserine antibody (1:500, Zymed Laboratories Inc.).

To detect phosphoserine bands, particular care was needed to block nonspecific binding of the anti-phosphoserine antibody. Western blots were blocked with Superblock blocking buffer (Pierce) for ~8 h and probed with anti-phosphoserine antibody in 10% bovine serum albumin in Tris-buffered saline (TBS) with 0.05% Tween 20 for 12 h. Blots were washed three times in TBS and twice in TBS-Tween 20 (5 min each) and then probed with goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, Inc.) (1:40,000 in 10% Superblock blocking buffer in TBS-Tween 20) for 40 min at room temperature. Blots were washed as described and exposed to x-ray film.

Specificity of the anti-phosphoserine antibody was demonstrated by treating samples with alkaline phosphatase (data not shown). Initially, RyR was immunoprecipitated from CHO cell lysate, and the immunoprecipitate was separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-phosphoserine antibody. A band was detected at ~560 kDa, indicating phosphorylated RyR. Because the band corresponding to the RyR ran at a higher molecular weight and was distinct from all of the other bands on the blot, in subsequent experiments this Western blot analysis was done using whole cell lysate. To further confirm that this band was the RyR, we routinely ran a lane of purified RyR1 (kindly provided by Dr. Susan Hamilton, Baylor College of Medicine, Houston, TX) as a positive control. This band ran at the same molecular weight as the immunoreactive band in each sample and showed a time-dependent increase in phosphorylation levels when the RyR1 standard was treated with the catalytic subunit of PKA (data not shown). The relative level of phosphorylation was determined by densitometric scan of the immunoreactive band of the same molecular weight as the RyR. Further confirmation that this band was RyR was its absence in the lane containing lysate from untransfected CHO cells. To control for variability in protein loading, the intensity of the phosphoserine-immunoreactive band was normalized by the intensity of the RyR-immunoreactive band. Total protein concentration was kept constant at 40 μg/lane for the phosphoserine Western blots.

Measurement of Caffeine-induced Ca^{2+} Transients—CHO cells expressing RyR were loaded at 37 °C with 1 μM Fura-2 acetoxymethyl ester at 48 h following transfection with mAKAP or mAKAP-P. After a 30-min incubation with the Fura-2, the cells were incubated at 37 °C in Krebs-Ringer buffer (140 mM NaCl, 5 mM KCl, 2 mM MgCl, 10 mM

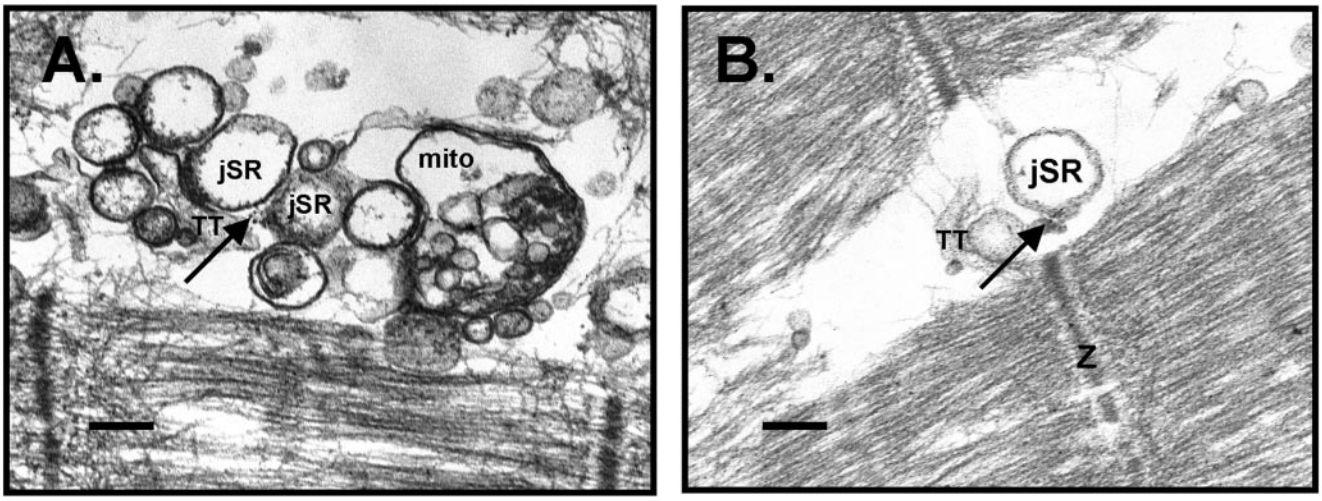

FIG. 1. **Indirect immunogold labeling of mAKAP in rat skeletal muscle.** mAKAP immunoreactivity (*arrow*) was associated with electron dense feet structures, which are indicative of RyR. *TT*, transverse tubule; *Z*, Z-line; *mito*, mitochondria. *Bar* = 225 nm.

HEPES, 12 mM glucose, and 2 mM $CaCl_2$, pH 7.4). Fluorescence measurements were obtained at excitation wavelengths of 340 and 380 nm and emission at 510 nm on individual cells using an inverted fluorescence microscope (Olympus IX-70) as described previously (4). Fluorescence spectra were continuously sampled at a frequency of 50 Hz and collected with a spectrofluorometer (Photon Technology International, Monmouth Junction, NJ) as described previously (30). Base-line intracellular Ca^{2+} levels were measured as the ratio of 340/380-nm fluorescence in individual cells incubated in Krebs-Ringer phosphate solution buffer. Cells were treated for 5 min with 10 μM forskolin in Krebs-Ringer buffer to activate adenylyl cyclase. Cells were then exposed to 10 mM caffeine in the presence of forskolin. Changes in amplitude of the caffeine-induced intracellular Ca^{2+} transient were determined for each cell by subtracting the base-line level of Ca^{2+} (average value determined over 1 min of base-line readings) from 1 min of measurements following caffeine addition.

RESULTS

Co-distribution of RyR1 and mAKAP at the Perinuclear Space—AKAPs target PKA to discrete areas within a cell. It is predicted that upon activation of the PKA pathway, PKA, which is tethered to an AKAP in the vicinity of local increases in cAMP, will preferentially be activated, compared with PKA not anchored at these sites. AKAPs may also function by bringing PKA closer to a PKA substrate. Thus, we predict that once PKA is activated, anchoring of PKA to its substrate should regulate the extent of phosphorylation of that substrate.

Both PKA and mAKAP co-immunoprecipitate with RyR2 (16) and RyR1 (17). Thus, we first determined at the electron microscope level whether mAKAP is co-localized with RyR in native skeletal muscle. mAKAP is localized to two different subcellular regions. It is distributed in the perinuclear region in rat neonatal ventricular myocytes and adult cardiac tissue (13, 14). Also, by confocal fluorescence microscopy, we previously showed that mAKAP is found near the jSR/transverse tubule in cardiac muscle cells (13), an area enriched in RyR. To examine mAKAP localization at higher resolution and to determine whether mAKAP is located next to the RyR, we used immunogold electron microscopy to determine the location of mAKAP and RyR in gently disrupted skeletal muscle. mAKAP was located in close proximity to the electron dense periodic foot structure, which has been shown to be the RyR (Fig. 1) (31). Thus, not only does mAKAP directly bind RyR in *in vitro* experiments, it also co-localizes with the RyR at the jSR in native cardiac tissue.

To investigate the effect of mAKAP on the phosphorylation state of the RyR and its effect on RyR function, we chose to study these proteins in a cell culture system. CHO cells were

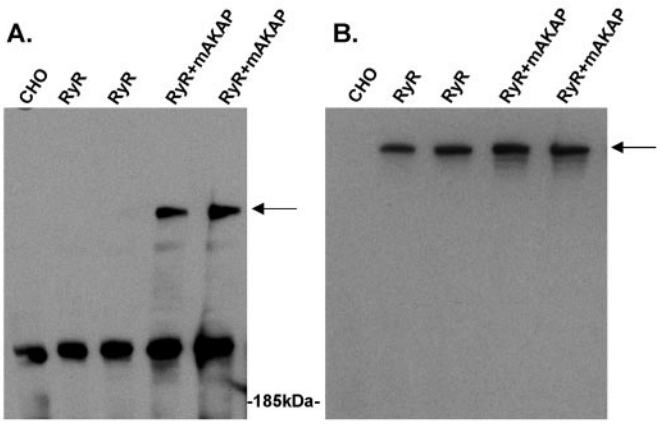

FIG. 2. **Expression of mAKAP and RyR1 in CHO cells.** CHO cell lysate (50 μg) from cells transfected with mAKAP-ECFP and RyR1-EGFP was subjected to Western analysis as described under "Experimental Procedures." The Western blot was first probed with an anti-mAKAP antibody (*A*) and then reprobed with anti-RyR antibody (*B*). *Arrows* point to mAKAP on *blot A* and RyR1 on *blot B*. The *lower band* on *blot A* represents nonspecific labeling by the secondary antibody. The molecular mass marker at 185 kDa is indicated between the two blots.

transfected with RyR1 (CHO-RyR1). Overexpressed RyR1 is targeted to the endoplasmic reticulum (ER) and gates Ca^{2+} release from the ER, thus mimicking the regulation of Ca^{2+} release from the SR of native muscle cells (24, 32). Using Western blot analysis, we determined that full-length mAKAP and RyR were expressed in transfected CHO cells (Fig. 2). Untransfected CHO cells did not express mAKAP (Fig. 2*A*) or RyR1 (Fig. 2*B*).

mAKAP Expression Increases PKA-dependent RyR1 Phosphorylation—To determine the effect of expression of mAKAP on the phosphorylation state of RyR, CHO-RyR1 cells were transiently transfected with either mAKAP or mAKAP-P. We hypothesized that upon stimulation of PKA, the level of RyR phosphorylation would be greater in cells expressing mAKAP than mAKAP-P because mAKAP-P should not localize PKA near the RyR. Upon PKA stimulation compared with cells expressing mAKAP-P, RyR phosphorylation was increased by 42.4 ± 6.6% (mean ± S.E.) (n = 4) in cells expressing mAKAP ($p < 0.05$) (Fig. 3).

The anti-phosphoserine antibody is selective for phosphorylated serine residues. This antibody could then be used to detect PKA-dependent phosphorylation of the RyR, because the only

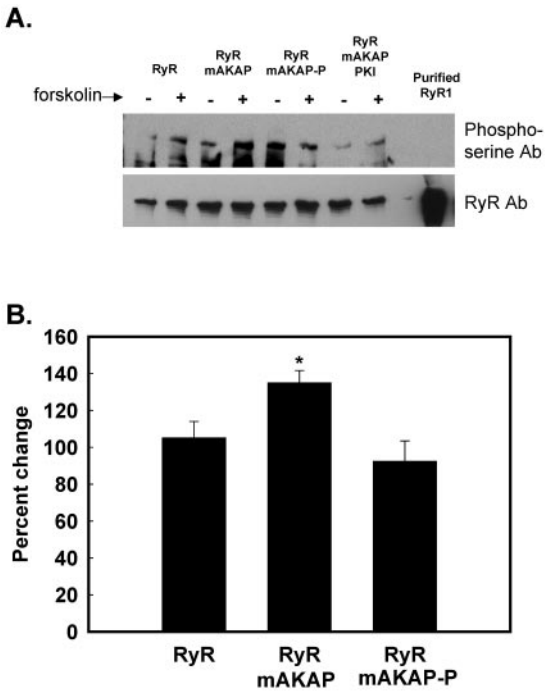

FIG. 3. **Effects of PKA activation on RyR phosphorylation in the presence or absence of mAKAP or mAKAP-P.** *A*, cells expressing RyR1 in the presence or absence of mAKAP or mAKAP-P were treated with 10 μM forskolin for 5 min. Cell lysate was subjected to Western blot analysis and probed for phosphoserine immunoreactivity (*top blot*). The blot was reprobed with a RyR antibody to ensure equal amounts of protein (*bottom blot*). −, no forskolin treatment; +, forskolin treatment. Purified RyR1 (kindly provided by Dr. Susan Hamilton, Baylor College of Medicine, Houston, TX). *B*, immunoreactive bands were analyzed using densitometry, and the relative intensity of the phosphoserine band was divided by that of the corresponding RyR band in each lane to control for variability in protein levels. The percent change in forskolin-induced phosphorylation was determined by dividing the relative intensity (as determined above) of the forskolin-treated group by that of the corresponding group that was not treated with forskolin and multiplying by 100. *, significantly different $p < 0.05$ ($n = 4$).

known phosphorylation site for PKA on the RyR is at serine 2843 (33). Ca^{2+}-calmodulin-dependent protein kinase (CaM kinase) has also been reported to phosphorylate RyR on this same serine residue (33); however, in our experiments, the addition of the PKA-specific inhibitor PKI attenuated RyR1 phosphorylation in CHO-RyR1 cells expressing mAKAP that were stimulated with forskolin (Fig. 3*A*). In addition, the activation of the adenylyl cyclase/PKA pathway by forskolin did not increase intracellular Ca^{2+} (Fig. 4), thus forskolin treatment should not result in Ca^{2+}-dependent activation of CaM kinase. Thus, we can conclude that the forskolin-dependent increase in RyR1 phosphorylation is the result of activation of PKA.

Expression of mAKAP Increases PKA-dependent Ca^{2+} Release by RyR1 from Internal Stores—Since we were able to demonstrate that RyR1 phosphorylation is greater in CHO-RyR1 cells expressing mAKAP as compared with CHO-RyR1 cells expressing mAKAP-P, we wished to determine whether expression of mAKAP (*versus* mAKAP-P) influences RyR-dependent Ca^{2+} release from the ER. PKA-dependent phosphorylation has previously been shown to increase Ca^{2+} efflux from the SR via RyR2 (20). For example, in pancreatic β-cells, the caffeine-dependent Ca^{2+} transient (indicative of Ca^{2+} release from the ER) is significantly increased in the presence of forskolin (20). There was no significant change in base-line cytosolic Ca^{2+} when CHO cells were incubated with 10 μM forskolin *versus* no forskolin as evident in the representative traces (Fig. 4). However, treatment with 10 mM caffeine in the presence of 10 μM forskolin significantly increased the amplitude of the intracellular Ca^{2+} transient in CHO-RyR1 cells expressing mAKAP compared with cells expressing mAKAP-P (Fig. 5).

DISCUSSION

The results of this study show that by anchoring PKA in close vicinity to RyR1, mAKAP regulates RyR function. PKA is targeted to RyR1 (17), and mAKAP serves as the anchoring protein to link this kinase to RyR1 (17). Our study is the first to demonstrate that mAKAP plays a key role in regulating PKA-dependent changes in RyR1 phosphorylation and Ca^{2+} efflux from internal stores, thus indicating an important regulatory role of mAKAP in RyR1 function. These findings are consistent with previous studies showing that disruption of the AKAP-RII interaction by the addition or expression of a competing RII-binding peptide decreases PKA-dependent phosphorylation and/or results in change in PKA-dependent cellular function (2, 4, 9, 10).

Extrapolating these results to the role of PKA-dependent RyR phosphorylation in muscle, we predict that in response to activation of adenylyl cyclase, increases in cAMP near the jSR would rapidly elicit increased RyR phosphorylation and, as a result, greater Ca^{2+} efflux through this channel. In cardiac muscle, PKA-dependent activation of RyR2 could be rapidly reversed as a result of hydrolysis of cAMP by mAKAP-bound PDE4D3, thus turning off the PKA-dependent response (34). This may also be the case in skeletal muscle, although it remains to be established. It is also likely that phosphorylation of RyR is also reversed by protein phosphatase 1 in both skeletal and cardiac muscles and by protein phosphatase 2A (PP2A) in cardiac muscle, because these phosphatases are tethered to RyR by specific targeting proteins (17). Therefore, our results indicate that mAKAP is likely to play an important role in regulating the function of this channel and controlling Ca^{2+} release from the SR.

The role for PKA-dependent RyR phosphorylation in the regulation of muscle contraction has been more thoroughly investigated in cardiac than in skeletal muscle. Suko *et al.* (33) showed that PKA as well as cGMP-dependent protein kinase (PKG) and Ca^{2+}-calmodulin-dependent protein kinase phosphorylate RyR1 at serine 2843. An analogous phosphorylation site at serine 2809 exists in RyR2 (35). Previous studies have indicated that the level of PKA-dependent phosphorylation is greater in cardiac RyR2 than in skeletal RyR1 (36, 37). These authors concluded that PKA-dependent phosphorylation plays a greater role in the regulation of excitation-contraction coupling in cardiac muscle compared with skeletal muscle. In agreement with this idea, PKA-dependent phosphorylation of RyR1 was not required for excitation-contraction coupling in skinned fast twitch skeletal muscle cells (38). However, another group reported previously that PKA-dependent phosphorylation increased the open probability of skeletal RyR1 by removing the Mg^{2+} block from the channel (22), thus potentiating Ca^{2+} efflux through the channel. Phosphorylation of RyR1 may be necessary for channel opening in response to activation by the DHPR (39) as well as for increasing Ca^{2+} release from the SR (21). Thus, several groups have determined that phosphorylation of RyR1 regulates its function by enhancing Ca^{2+} efflux through this receptor.

Many proteins including two different AKAPs have been discovered in the ~12-nm space between the T-tubule and jSR membrane (Fig. 6). The cytoplasmic II-III loop of the α_1-subunit of the skeletal DHPR located at the T-tubule has also been shown to bind to a region of the RyR1 (40–42). It has been proposed that there is a direct link between the DHPR and

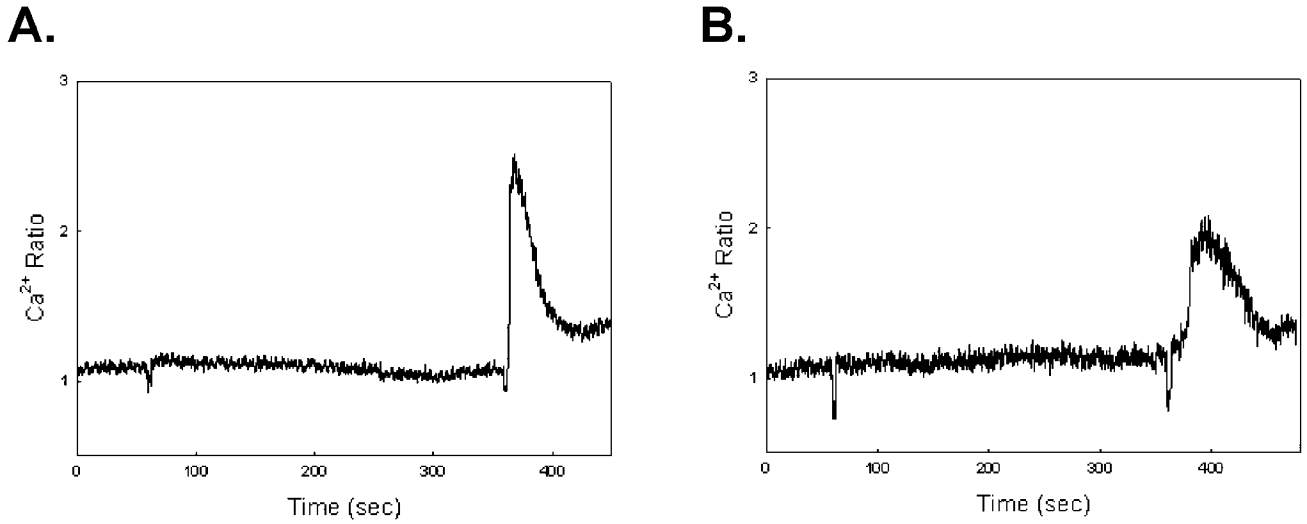

FIG. 4. **Representative Ca^{2+} traces of the effect of mAKAP *versus* mAKAP-P on PKA-dependent caffeine-induced Ca^{2+} release from the ER.** Intracellular Ca^{2+} transients were measured in CHO-RyR1 cells expressing mAKAP (*A*) or mAKAP-P (*B*) using Fura-2 as described under "Experimental Procedures." Medium was replaced with 10 μM forskolin-containing medium at 1 min and then with 10 mM caffeine + 10 μM forskolin-containing medium at 6 min as indicated by the *solid* and *dashed arrows*, respectively.

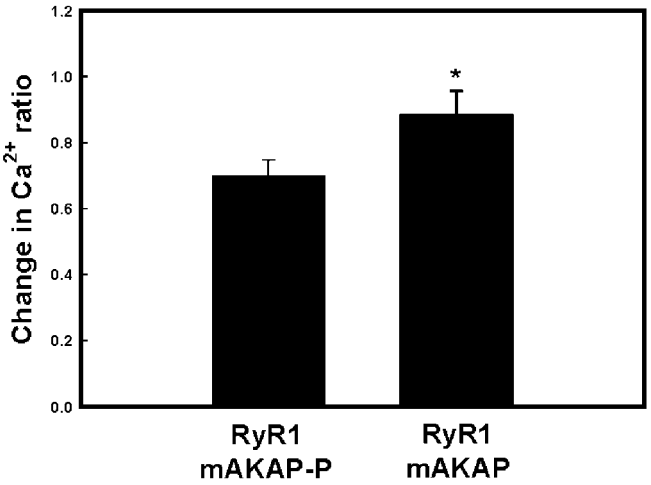

FIG. 5. **mAKAP tethering of PKA increases PKA-dependent caffeine-induced Ca^{2+} release from the ER.** Intracellular Ca^{2+} transients were measured using Fura-2 as described under "Experimental Procedures." Cells were treated with forskolin (10 μM) for 5 min. Ca^{2+} efflux was then stimulated with caffeine (10 mM) + forskolin (10 μM). The change in intracellular Ca^{2+} accumulation was determined by subtracting the peak increase in the 340/380 nm excitation ratio following caffeine treatment by the base-line reading (see Fig. 4). *, significantly different ($p < 0.05$).

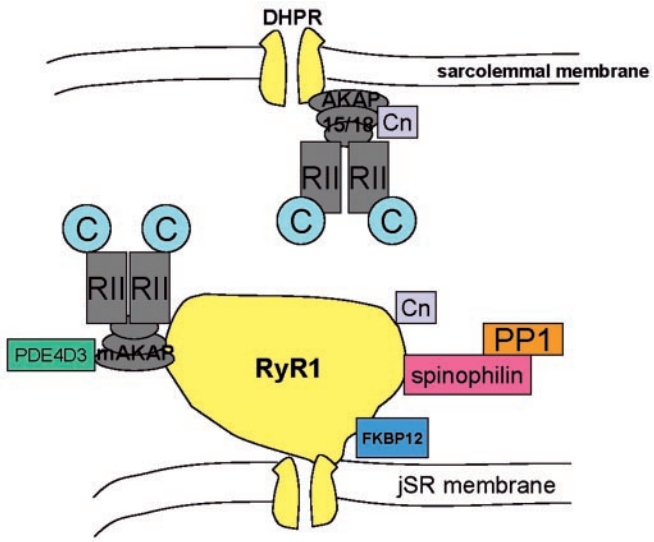

FIG. 6. **Schematic of the T-tubule/junctional sarcoplasmic reticulum.** *Cn*, calcineurin (PP2B); *FKBP*, FK-binding protein. Sources for the interaction between the proteins listed above with their respective binding partners are cited in the text.

RyR1 that mediates the voltage-dependent activation of RyR1 in skeletal muscle (43, 44). Interestingly, it also has been proposed that the DHPR is associated with another AKAP, AKAP15/18 (45–47). An interesting question that emerges from these studies is why two different AKAPs are located in this small gap between the T-tubule and jSR membrane and whether PKA targeted to the DHPR could phosphorylate the RyR or vice versa. Using surface plasmon resonance, we showed that mAKAP has a greater affinity for RII than AKAP15/18 and that this affinity for RII depends on the phosphorylation state of RII (48). Accordingly, RII may be preferentially targeted to either mAKAP or AKAP15/18 depending on its phosphorylation state, thus providing a mechanism to modulate PKA-dependent signal transduction.

It is not known whether PKA targeted to the DHPR could phosphorylate the RyR or whether PKA targeted to RyR could phosphorylate the DHPR. Whereas evidence suggests that the catalytic (C) subunit can diffuse through the cytosol and phosphorylate substrates that are not in the immediate vicinity of an AKAP (3), the distances between AKAP15/18 and the PKA phosphorylation site on RyR or mAKAP and the PKA phosphorylation site on DHPR are not known. The RyR is a large protein with each of its four subunits being ~500 kilodaltons. This receptor itself may hinder the diffusion of the C-subunit from PKA tethered to mAKAP to the DHPR. Aside from this rather bulky receptor blocking the diffusion of the C-subunit, several other accessory proteins are bound to the RyR and also may impede the movement of the C-subunit to another substrate. In fact, the subsarcolemmal space has been referred to as a "fuzzy space" where ion concentrations may be significantly different from ion concentrations in the bulk cytoplasm and where diffusion may be restricted (49–52).

Similar to AKAP15/18 or mAKAP, other proteins have been found to be targeted to both the DHPR and the RyR in skeletal muscle. For example, in skeletal muscle, calcineurin (PP2B) is associated with AKAP15/18 (53) as well as with RyR1 (23). This

phosphatase could then regulate the phosphorylation state of both the DHPR and RyR1. Calmodulin was one of the first protein observed to interact with RyR1 and RyR2 (54), and it may activate or inhibit the channel depending on the concentration of Ca^{2+} (54–56). Calmodulin also binds the C-terminal tail of the DHPR and plays a role in regulating the function of this receptor (56). Thus, the tethering of specific proteins including AKAPs to both the DHPR and the RyR may regulate signaling events in this highly specialized region.

In summary, our current study investigated the role of mAKAP targeting of PKA in PKA-dependent Ca^{2+} regulation. Our results indicate that mAKAP co-localizes with RyR1 in skeletal muscle. We show that mAKAP-dependent anchoring of PKA increases the phosphorylation of RyR1 in the presence of elevated levels of cAMP. Interestingly, when mAKAP tethers PKA to RyR1, the activation of PKA regulates the function of the RyR by increasing caffeine-induced Ca^{2+} efflux through this receptor. Therefore, since Ca^{2+} efflux through the RyR regulates the extent of muscle contraction, our findings suggest that modulation of this Ca^{2+} channel may regulate not only cardiac muscle function, as is well established, but may also play a role in skeletal muscle contractility.

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