mAKAP and the ryanodine receptor are part of a multi-component signaling complex on the cardiomyocyte nuclear envelope

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SUMMARY

The physical association of regulatory enzymes and ion channels at relevant intracellular sites contributes to the diversity and specificity of second messenger-mediated signal transduction in cells. mAKAP is a scaffolding protein that targets the cAMP-dependent protein kinase A and phosphodiesterase type 4D3 to the nuclear envelope of differentiated cardiac myocytes. Here we present data that the mAKAP signaling complex also includes nuclear envelope-resident ryanodine receptors and protein phosphatase 2A. The ryanodine receptor is the major cardiac ion channel responsible for calcium-induced calcium release from intracellular calcium ion stores. As demonstrated by a combination of immunohistochemistry and tissue fractionation, mAKAP is targeted specifically to the nuclear envelope, whereas the ryanodine receptor is present at both the sarcoplasmic reticulum and nuclear envelope intracellular membrane compartments. At the nuclear envelope, a subset of cardiac ryanodine receptor is bound to mAKAP and via the association with mAKAP may be regulated by protein kinase A-mediated phosphorylation. By binding protein kinase A and ryanodine receptor, mAKAP may serve as the scaffold for a cAMP- and calcium ion-sensitive signaling complex.

Key words: mAKAP, Protein kinase A, cAMP, Ryanodine receptor, Nuclear envelope, Heart

INTRODUCTION

Extracellular stimuli influence cells in part by the specific induction of small diffusable second messengers such as cAMP and calcium ions (Ca^{2+}). These second messengers regulate various cellular processes by activation of signaling enzymes, including protein kinases that phosphorylate target substrates (Hunter, 2000). In the heart, stimulation of adrenergic receptors increases cardiac output by elevating intracellular cAMP and activation of cAMP-dependent protein kinase (PKA). Physiologic PKA substrates in the heart include the L-type calcium channel, phospholamban, the ryanodine receptor (RyR), and the contractile protein troponin. Specificity in signaling is partly due to the intrinsic substrate specificity of protein kinases. However, it has been long recognized that activation of PKA by different extracellular stimuli causes the phosphorylation of different subsets of PKA substrates, independently of substrate affinity (Steinberg and Brunton, 2001). To achieve this specificity, PKA signaling may be refined by the spatial segregation of PKA pools and relevant substrates by A-kinase anchoring proteins (AKAPs) that are targeted to discrete intracellular locations (Colledge and Scott, 1999).

The PKA holoenzyme is composed of two regulatory (R) and two catalytic (C) subunits, which dissociate upon cAMP binding (Scott, 1991). PKA holoenzyme is sequestered in pools by constitutive binding of the R-subunit homodimer to AKAPs (Colledge and Scott, 1999). For example, AKAP18 is targeted to the cardiac myocyte plasma membrane, where it binds PKA that can phosphorylate and activate the L-type calcium channel (Fraser et al., 1998; Gray et al., 1998). AKAPs often serve as scaffolds for multi-protein complexes that include kinase substrates and other signaling enzymes. AKAP79/150 is one such scaffolding protein that associates in brain with PKA, protein kinase C, the protein phosphatase calcineurin, and the β_2-adrenergic receptor (Fraser et al., 2000). Rather than the concentration or overall abundance of signaling enzymes and substrates, it is the localization of a particular set of enzymes via association with a targeted scaffolding protein that confers specificity in function (Pawson and Nash, 2000; Pawson and Scott, 1997).

mAKAP (muscle A-Kinase Anchoring Protein) is a 255 kDa scaffolding protein present on the nuclear envelope (NE) of myocytes in heart and skeletal muscle that can bind PKA and phosphodiesterase type 4D3 (PDE4D3) (Dodge et al., 2001; Kapiloff et al., 1999). mAKAP targeting has been studied using recombinant fragments fused to green fluorescent protein (GFP). mAKAP is localized in the differentiated cardiomyocyte NE by two independent targeting domains, amino acid (aa) residues 772-915 and 915-1065, which contain spectrin-like repeat motifs (Kapiloff et al., 1999). mAKAP targeting is saturable, and endogenous mAKAP can be displaced by overexpression of an mAKAP fragment containing the targeting domains. mAKAP binds PKA via a

putative amphipathic helix comprising aa residues 2055-2072, and PKA binding can be disrupted by substitution of isoleucine residue 2062 with a proline residue. This is consistent with structural data regarding the AKAP-RIIα interaction that the N-terminal domains of the PKA type II R-subunit homodimer form an X-type, four-helix bundle dimerization motif containing a hydrophobic groove that accommodates an AKAP amphipathic α-helix (Newlon et al., 2001). Through binding to a site within mAKAP residues 1286-1831, a rolipram-inhibited, cAMP-specific PDE4D3 is also associated with the mAKAP-PKA complex in heart (Dodge et al., 2001). PDE4D3-catalyzed degradation of cAMP is enhanced by cAMP-dependent PKA phosphorylation, thereby constituting a local, negative feedback loop to modulate NE-targeted, cAMP-dependent signaling.

mAKAP was initially identified by its ability to bind PKA (McCartney et al., 1995). Despite an understanding of its intracellular location and its association with PDE4D3, the function of mAKAP at the NE is yet uncertain. Using a candidate-directed approach, we have identified an association of mAKAP with the ryanodine receptor (RyR) and protein phosphatase 2A (PP2A). As shown below, mAKAP and RyR overlap in intracellular distribution at the NE of cardiac myocytes, a double membrane structure separating the cytoplasm, the perinuclear space and the nucleus. The perinuclear space holds a discrete store of intracellular Ca^{2+} that may be released into the surrounding area by NE-associated ion channels such as the RyR, with potential effects on gene expression (Abrenica and Gilchrist, 2000; Adebanjo et al., 1999; Adebanjo et al., 2000; Badminton et al., 1996; Chawla et al., 1998; Franco-Obregon et al., 2000; Gerasimenko et al., 1995; Malviya and Rogue, 1998; Rogue et al., 1998). Tetrameric with subunits of 560 kDa, the RyR is a high conductance Ca^{2+} channel, tightly regulated by multiple co-factors, notably Ca^{2+} itself, and by phosphorylation, including PKA-catalyzed phosphorylation (MacKrill, 1999). PP2A is a phospho-serine/threonine protein phosphatase that is involved in the regulation of many signaling pathways (Millward et al., 1999). PP2A has three subunits, a catalytic C-subunit and a scaffolding A-subunit that comprise a constitutive, core heterodimer and one of several possible regulatory B-subunits. We now describe the association of PP2A and RyR with the mAKAP complex, an assembly that is likely to be important to the integration of cAMP and Ca^{2+} signaling to the myocyte nucleus.

MATERIALS AND METHODS

Materials

Antibodies were as follows: anti-RyR, MA3-916 (monoclonal, Affinity Bioreagents); anti-PP2A catalytic subunit, P47720 (monoclonal, Transduction Laboratories); anti-PKA RIIα subunit, P55120 (monoclonal, Transduction Laboratories); anti-PKA catalytic subunit, P73420 (monoclonal, Transduction Laboratories); anti-phospholamban (PLB), MA3-922 (monoclonal, Affinity Bioreagents); anti-PDE4D, PD4-401AP (rabbit polyclonal, FabGennix); anti-lamina associated polypeptide 2 (LAP2), L74520 (monoclonal, Transduction Laboratories); and anti-calsequestrin, 06-382 (rabbit polyclonal, Upstate Biotechnology). Anti-mAKAP VO54 and VO56 rabbit polyclonal antibodies were affinity-purified using agarose beads (Affigel, Biorad) previously absorbed with a recombinant rat mAKAP fragment comprising aa residues 1401-2314, as previously described (Kapiloff et al., 1999). A full-length rabbit RyR type II cDNA was the gift of Kurt Beam (Colorado State University, Fort Collins, CO). A bacterial expression vector for murine PP2A A-subunit-GST (glutathione S-transferase) fusion protein, was the gift of Kerry S. Campbell (Fox Chase Cancer Center, Philadelphia, PA).

Immunohistochemistry of human heart tissue

Anonymous samples of paraffin-embedded normal human heart tissue were provided by the Cancer Pathology Shared Resource of the Oregon Cancer Center. 5 μm sections of these samples were prepared on Fisherbrand Plus slides (Fisher Scientific). Following de-paraffinization and rehydration, the slides were subjected to epitope retrieval by heating in a vegetable steamer (Black & Decker Inc.) containing 1 M Tris, pH 10, for 20 minutes and then cooling to room temperature. Treated slides were then washed with PBS (120 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate salts) containing 0.05% Tween (Sigma) and used for immunohistochemistry.

Antibodies were diluted into dilution buffer (PBS, 1% bovine serum albumin (BSA), 0.1% Tween 20, 0.1% sodium azide). Tris buffered saline (TBS; 10 mM Tris, pH 7.5, 150 mM NaCl) was used for all wash steps. Following a 10 minute incubation in dilution buffer, primary antibody (4 μg/ml VO56 affinity-purified antibody or rabbit whole IgG) was added for 45 minutes followed by washing. The slides were treated with quench solution (methanol, 6% H_2O_2) for 10 minutes, washed again, and then incubated with Envision anti-rabbit secondary reagent (Dako) for 30 minutes. After additional washes, premixed DAB solution (K3466, Dako) was added to the slides and allowed to react for 10 minutes. Slides were counterstained with hematoxylin prior to dehydration and coverslipping.

Immunoprecipitation from heart extracts

Rat hearts (Pel-freeze) were washed twice with cold PBS and then disrupted using a Polytron homogenizer at half-speed for 15 seconds in Buffer A (50 mM Hepes, pH 7.4, 10% glycerol, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM 4-(2-Nethyl)benzenesulfonyl fluoride (AEBSF), 1 mM benzamidine, 1 mM dithiothreitol (DTT), 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol-bis(β-aminoethyl ether), 25 mM sodium fluoride, 40 mM β-glycerophosphate, and 1 mM sodium pyrophosphate). A low-speed pellet was obtained by centrifugation of whole heart extract at 13,000 ***g*** for 5 minutes. This pellet was resuspended in Buffer A with 100 mM NaCl and 0.5% Triton X-100, mixed for 10 minutes at 4°C, and then a solubilized protein supernatant was generated by centrifugation at 20,000 ***g*** for 10 minutes. Solubilized protein supernatant was mixed at 4°C for 3 hours with 20 μl preimmune or VO54 anti-mAKAP immune antiserum previously bound to 20 μl protein-G-agarose (Upstate Biotechnology). Beads were washed for 5 minutes three times with Buffer A with 100 mM NaCl and 0.5% Triton X-100 before resuspension in sample buffer (12.5 mM Tris-HCl, pH 6.8, 1% mercaptoethanol, 2% glycerol, 0.4% sodium dodecyl sulfate, 6 μg/ml bromophenol blue).

Immunoblotting

Samples were size-fractionated by SDS-PAGE on 3% acrylamide stacking phase, 5% or higher percentage acrylamide resolving phase gels (Laemmli, 1970). 5% acrylamide resolving phase gels were transferred to nitrocellulose on a Biorad Semi-dry Transfer Unit in the absence of methanol, while 8-12% acrylamide resolving phase gels were transferred using a Biorad Liquid Transfer Unit in the presence of 10% methanol. Blots were blocked in 0.1% Blotto (0.1% BSA, 5% nonfat dry milk, TBS, 0.05% sodium azide) for 30 minutes and incubated with primary antibody diluted in 0.1% Blotto (polyclonal antibodies) or TTBS (TBS with 0.03% Tween; monoclonal antibodies) overnight at room temperature. Blots were washed four times for five minutes with TTBS, before incubation for 1 hour with

1:50,000 dilution of horseradish peroxidase-conjugated donkey anti-IgG antibody (Jackson Laboratories) in TTBS. After washing, bound antibody was detected with chemiluminescent substrate (Supersignal products, Pierce). Molecular weight markers include myosin (200 kDa), IgG heavy chain (48 kDa) and Kaleidoscope Prestained Standards (Biorad).

Ventriculocyte immunocytochemistry

Rat neonatal ventriculocytes were prepared as previously described (Kapiloff et al., 1999) and cultured on dual-well chamberslides coated with 1% gelatin and 1 mg/ml laminin solution at a density of about 100,000 myocytes/cm^2. After one day in plating medium (Dulbecco's Modified Eagle Medium (DMEM) with 17% Media 199, 1% penicillin/streptomycin solution (Gibco/BRL), 10% horse serum, and 5% fetal bovine serum (FBS)), cells were incubated for two days in 80% DMEM, 20% Media 199 and 100 μM phenylephrine. For staining, cells were washed twice with PBS, fixed for 10 minutes in 3.7% formaldehyde in PBS, washed once with PBS, permeabilized with 0.3% Triton X-100 in PBS for 10 minutes, washed again with PBS and then blocked in PBS with 1% horse serum, 0.2% BSA. Slides were incubated with primary antibody (1 μg/ml) in blocking solution for 1 hour and washed several times with blocking solution. Slides were next incubated for 1 hour with Cy5 or FITC-conjugated donkey secondary antibodies (Jackson Laboratories) and Rhodamine Phalloidin (Molecular Probes), washed several times with PBS, and then mounted with coverslips and Slofade anti-fade solution (Molecular Probes). Hoechst 33258 stain (10 μg/ml) was included in the last PBS wash in order to locate nuclei. Specific immunofluorescence was detected in successive focal planes by laser-scanning confocal microscopy on an MRC1024 Biorad UV/Vis System.

Heart subcellular fractionation

The following procedure is a modification of established procedures for the isolation of sarcoplasmic reticulum (SR) and nuclei from heart tissue (Meissner, 1974; Tata, 1974). Two rat hearts (Pel-freeze) were washed twice with cold PBS and then disrupted using a Polytron PT10/35 Generator at half-speed for 15 seconds in 20 ml Buffer B (10 mM Hepes, pH 7.4, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM AEBSF, 1 mM benzamidine, 5 mM EDTA) with 0.32 M sucrose. Whole heart homogenate was filtered through 2 layers and then 4 layers of cheesecloth, before low-speed centrifugation at 3800 ***g*** for 20 minutes. The supernatant fraction (S1) was clarified by centrifugation at 10,000 ***g*** for 20 minutes, before re-centrifugation at 100,000 ***g*** for 1 hour. The resulting pellet (P2 fraction), containing SR, Golgi apparatus and plasma membrane, was resuspended in 2 ml buffer B and 0.32 M sucrose. Purified SR was obtained from P2 fraction by sucrose step gradient centrifugation (8 parts 24%, 6 parts 40%, 2 parts 50% sucrose in 5 mM Hepes buffer) at 100,000 ***g*** for 90 minutes. Purified SR forms a layer at the interface between 24% and 40% sucrose.

The initial 3800 ***g*** pellet (P1), containing myofibrils, mitochondria and nuclei, was resuspended in 20 ml Buffer B with 2.4 M sucrose, and nuclei were sedimented by centrifugation at 50,000 ***g*** for 90 minutes. The nuclei-containing pellet was washed by resuspension in 1 ml buffer B and 0.32 M sucrose and repeat centrifugation at 3800 ***g*** for 20 minutes. The nuclei-containing pellet (Nuclei) was then resuspended in 1 ml buffer B and 0.32 M sucrose. mAKAP complex was immunoprecipitated from nuclei as above. Protein content was determined by a modification of the Lowry method (Lowry et al., 1951; Biorad DC Protein Assay kit).

Expression of full-length mAKAP and protein-protein interaction studies

A mAKAP cDNA encoding the full-length protein was subcloned from pEGFPN1 (Kapiloff et al., 1999) into the *Nhe*I and *Kpn*I sites in pCDNA3.1(–)mychis B (Stratagene) in order to introduce a myc tag fusion. The fusion cDNA was transferred into pTRE-shuttle vector (Clontech) using *Spe*I and *Afl*II restriction enzymes and recombinant adenovirus was produced using the Adeno-X Tet-Off Expression System (Clontech), according to the manufacturer's protocols. mAKAP-expressing adenovirus was used to infect HEK293 and COS-7 cells for the production of mAKAP protein. RyR GST-fusion proteins were produced in *E. Coli* BL21 DE3 RIL (Stratagene) by way of pGEX-4T expression vectors (Pharmacia Biotech) containing rabbit RyR type II cDNA fragments that were inserted by ligation of restriction enzyme digested PCR products generated with Pfu Turbo high-fidelity enzyme (Stratagene), a RyR II full-length cDNA, and oligonucleotide primers designed to keep the cDNA in frame. GST-fusion proteins were purified from bacterial extracts using glutathione resin as suggested by the manufacturer (Clontech).

HEK293 or COS-7 cells were placed into culture at 50% confluence in DMEM with 10% FBS and 1% penicillin/streptomycin solution. The next day, cells were infected with mAKAP-expressing adenovirus and Adeno-X Tet-Off virus as suggested by the manufacturer (Clontech). 24-48 hours later, cells were lysed in Buffer A with 100 mM NaCl and 0.5% Triton X-100 (without phosphatase inhibitors). Soluble proteins were separated by centrifugation at 20,000 ***g*** for 10 minutes, divided into aliquots and incubated at 4°C with glutathione resin previously bound with GST-fusion proteins. Beads were washed three times for 5 minutes at 4°C with lysis buffer before resuspension in sample buffer, separation by SDS-PAGE and immunoblotting as described above.

Protein kinase A assay

Rat hearts were homogenized as above using Buffer C (50 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.5% Triton X-100, 1 mM DTT, 10% glycerol, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM AEBSF, 1 mM benzamidine). Whole heart homogenate was cleared by centrifugation at 20,000 ***g*** for 10 minutes. mAKAP complex was immunoprecipitated as above using VO54 antiserum, and RyR complex using anti-RyR antibody. Beads containing immunoprecipitated complex were resuspended in 80 μl kinase buffer (50 mM MOPS, pH 6.8, 50 mM NaCl, 4 mM $MgCl_2$, 1 mM DTT, 1 mM cAMP, 20 μM U0126, 50 nM microcystin and 10 μM rolipram) and pre-incubated for 30 minutes at room temperature with and without 10 μM protein kinase inhibitor (PKI). Phosphorylation was initiated by addition of 5 μl [γ-^{32}P]ATP (7000 Ci/mmol, 13 μCi/μl). After incubation at 30°C for 30 minutes, reactions were stopped with 10 μl 5× sample buffer and 1 mM H_4PO_2. Kinase reactions were size-fractionated by SDS-PAGE and transferred to nitrocellulose as described above. Nitrocellulose filters were exposed to x-ray film and later incubated with antibody as described above. Control immunoprecipitations included addition of excess (100 μg) VO54 antigen (mAKAP residues 1401-2314) (Kapiloff et al., 1999) or use of non-immune immunoglobulin (2.5 μg IgG). The relative extent of phosphorylation was quantified by autoradiograph densitometry using a Biorad GS-700 Imaging Densitometer.

RESULTS

mAKAP is a nuclear envelope targeted cardiac protein

Previous studies with rat heart tissue and cardiomyocytes and GFP-fusion proteins suggested that mAKAP is localized at the cardiac NE through a domain containing spectrin-like repeats (Dodge et al., 2001; Kapiloff et al., 1999). In order to further those studies, 5 μM longitudinal sections of paraffin-embedded, human cardiac ventricle were stained with affinity-purified, VO56 anti-mAKAP antibody or control rabbit IgG antibody (Fig. 1). Sections were counterstained with

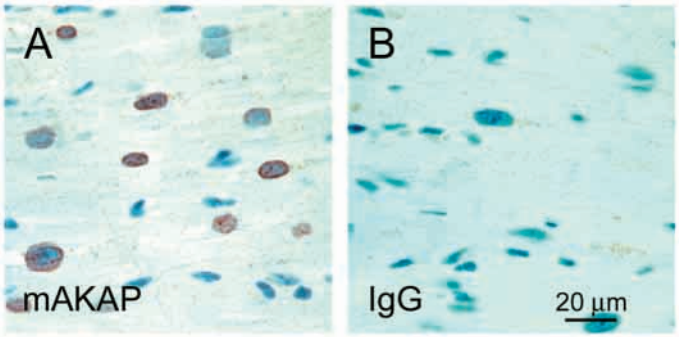

Fig. 1. The subcellular distribution of mAKAP in human heart tissue. Paraffin embedded, longitudinal sections of human heart tissue were stained with affinity-purified anti-mAKAP VO56 (A) or rabbit IgG (B) and Envision polymer horseradish-conjugated secondary antibody (brown stain). Nuclei were counter-stained with hematoxylin (blue). Scale is the same for each panel. Staining shown is representative of more than three separate experiments using sections from different individuals.

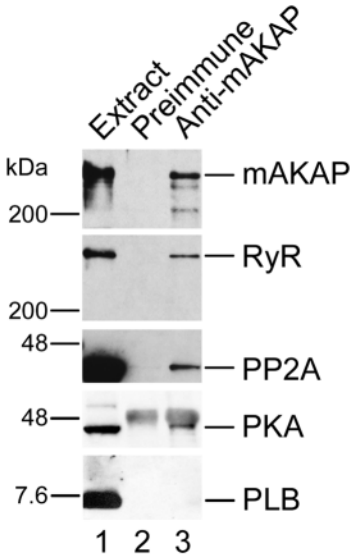

Fig. 2. Immunoprecipitation of the ryanodine receptor (RyR), protein phosphatase 2A (PP2A) and PKA with mAKAP antiserum. A 13,000 ***g*** pellet fraction of whole heart extract was solubilized with 0.5% Triton X-100 and 150 mM NaCl and cleared by repeat centrifugation. The solubilized pellet fraction was used for immunoprecipitation experiments with control preimmune serum (lane 2) or specific mAKAP VO54 antiserum (lane 3). Whole heart extract (5 µg, lane 1) and immunoprecipitates were subjected to SDS-PAGE and immunoblotting with purified mAKAP antibody and specific RyR, PLB, PP2A C-subunit and PKA C-subunit monoclonal antibodies. The migration of the respective protein and molecular weight markers are indicated for each panel. Each panel is representative of three separate experiments.

hematoxylin to detect nuclei, and the larger nuclei represented those present in myocytes. Specific mAKAP staining was limited to a ring surrounding myocyte nuclei (Fig. 1A). Faint diffuse staining was detected with nonspecific rabbit IgG, but no perinuclear staining was found (Fig. 1B). An identical staining pattern was detected in tissue sections derived from the explanted hearts of fifteen random human patients with end-stage cardiomyopathy, as well as with paraffin-embedded, mouse cardiac ventricle (data not shown).

RyR and PP2A are associated with an mAKAP complex

Given the large size of mAKAP, 2314 aa residues, and the precedence of other AKAPs serving as scaffolding proteins (Colledge and Scott, 1999), we were interested in whether the mAKAP complex included other signaling proteins. The NE is notable for containing proteins relating to the nuclear pore complex and proteins involved in Ca^{2+} cycling (Malviya and Rogue, 1998). We immunoprecipitated mAKAP complex from a solubilized, low-speed pellet fraction of whole heart homogenate enriched in nuclei (see Materials and Methods) and assessed the association of mAKAP with several known proteins (Fig. 2). RyR and PP2A C-subunit were co-immunoprecipitated with anti-mAKAP VO54 antiserum in conjunction with PKA catalytic subunit and mAKAP itself (Fig. 2, lane 3). PLB, a well-characterized PKA substrate involved in the regulation of Ca^{2+} re-uptake (Frank and Kranias, 2000), was not detected in VO54 immunoprecipitates and serves as a negative control for this experiment. In addition, none of these proteins was precipitated with preimmune antiserum (Fig. 2, lane 2). The lower molecular weight bands in lane 3 of the mAKAP blot were variable and represent protein degradation products, and the 48 kDa bands present in lanes 2 and 3 of the PKA blot represent IgG heavy chain present in the immunoprecipitation reactions. Other proteins that we did not detect in anti-mAKAP immunoprecipitates included SERCA2A, protein phosphatase 1 (PP1), and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (data not shown). These data suggest that mAKAP serves as a scaffold for a complex that, in addition to the phosphodiesterase PDE4D3 and the kinase PKA, includes the protein phosphatase PP2A and the Ca^{2+} stores release channel RyR.

RyR, PKA and mAKAP at the nuclear envelope

Most research on the RyR has been conducted in the context of SR excitation-coupling. Therefore, we were interested in whether mAKAP, RyR and PKA were in part co-distributed at the cardiomyocyte nuclear envelope (Fig. 3). Rat neonatal ventriculocytes were dissociated and cultured under conditions in which they exhibited spontaneous contractions. These cells were fixed, stained using affinity-purified VO54 anti-mAKAP antibody (Fig. 3A,E), anti-RyR monoclonal antibody (Fig. 3B), anti-PKA RIIα-subunit monoclonal antibody (Fig. 3F) and rhodamine phalloidin which detects F-actin in myofibrils (Fig. 3D,H,L, red channel), and studied by fluorescent confocal microscopy. mAKAP protein was detected exclusively at the NE, consistent with the staining found in human heart tissue (Fig. 1) and with previously published results (Dodge et al., 2001; Kapiloff et al., 1999). As found in other recent reports (Adebanjo et al., 1999; Tuvia et al., 1999), RyR staining displayed a periodic punctate pattern within the cytoplasm, indicative of the staining of internal membranes, including both SR and NE (Fig. 3B). RIIα staining was diffusely cytoplasmic, as expected by its presence in multiple intracellular compartments (Fig. 3F) (Fink et al., 2001). Composite images repeatedly showed significant overlap of RyR, RIIα and mAKAP staining solely

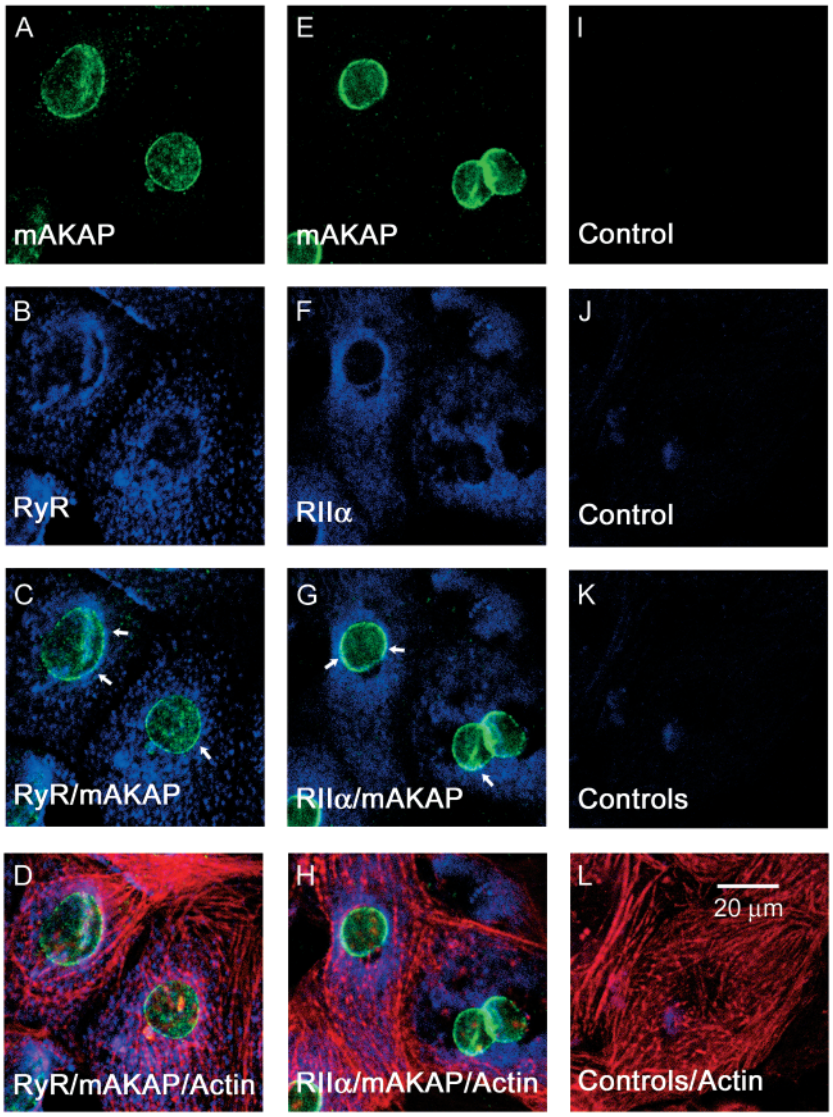

Fig. 3. Immunocytochemistry reveals the presence of mAKAP, RyR and PKA at the cardiomyocyte nuclear envelope. Primary cultures of rat neonatal ventriculocytes, maintained at high density in media containing 100 μM phenylephrine and exhibiting spontaneous contractile activity, were stained with anti-mAKAP VO54 polyclonal antibody (mAKAP, green, A,E), anti-RyR monoclonal antibody (RyR, blue, B), anti-PKA-RIIα subunit monoclonal antibody (RIIα, blue, F), rhodamine phalloidin, which detects F-actin in myofibrils (actin, red, D,H,L), mouse IgG (control, blue, J), or no primary antibody (control, green, I). FITC and Cy5-conjugated donkey anti-mouse and rabbit IgG secondary antibodies were used for specific detection. Panels C,G,K are composites of the preceding two images, and panels D,H,L are the same as panels C,G,K, except with the addition of the rhodamine channel. Nuclei, identified using Hoechst stain, occupied the area outlined by mAKAP staining (not shown). All images were acquired by confocal fluorescent microscopy and are presented at the same magnification as indicated by the bar in panel L. Staining shown is representative of greater than three separate experiments.

at the location of the NE (Fig. 3C,G). Important negative controls included the subtraction of primary antibody (Fig. 3I) or the substitution of primary antibody with nonspecific IgG (Fig. 3J), since nonspecific IgG will lightly stain striated muscle cells artificially.

Taken together, these images supported the hypothesis that a pool of mAKAP, RyR and PKA come together at the NE to form a signaling complex. Further support for this hypothesis was generated by subcellular fractionation of rat heart tissue (Fig. 4A). Hearts were disrupted in a neutral, isotonic buffer by mild homogenization (WH) and subjected to low-speed centrifugation to separate soluble proteins and microsomes (S1), including SR, from insoluble materials such as myofibrils, mitochondria and nuclei (P1). Nuclei were then purified from P1 fraction by sedimentation centrifugation. The S1 fraction was further cleared by medium-speed centrifugation, before microsomes (SR, plasma membrane and fragments of other organelles) were pelleted by ultra-centrifugation (P2). Purified SR was generated from the P2 fraction by sucrose-gradient equilibrium centrifugation (Fig. 4B, lane 3). The quality of heart fractionation was assessed by the specific enrichment of the nuclear envelope protein LAP-2 (Furukawa et al., 1998) in nuclear fractions (Fig. 4A, LAP-2, lane 5) and by the specific enrichment of the SR Ca^{2+}-binding protein calsequestrin (Tharin et al., 1996) in P2 fractions (Fig. 4A, Calsequestrin, lane 4). In addition, initial homogenates were routinely screened by microscopy for the presence of nuclear disruption (not shown).

Immunoblotting of the various heart fractions (5 μg protein each) revealed that RyR was present in all fractions tested, including P2 (containing SR) and purified nuclei (Fig. 4A, RyR). By contrast, mAKAP was present only in the NE-containing fractions, P1 and purified nuclei, and specifically not in the SR-containing fractions, S1 and P2 (Fig. 4A, mAKAP). Comparison of a larger quantity of purified SR and nuclear protein (25 μg) and longer chemiluminescent exposure confirmed that mAKAP was exclusively detectable in nuclear fractions (Fig. 4B, lanes 2,3). The lower molecular weight bands on the mAKAP and LAP-2 blots represent degradation products that were variable in the preparations (Fig. 4A,B). Although mAKAP was not present in SR, interestingly, a potential AKAP of about 140 kDa M_r was detected in SR fractions by RIIα overlay assay (Carr and Scott, 1992), the same size as one of the most prominent bands revealed by overlay assay of whole heart homogenate (data not shown). To confirm the existence of a nuclear pool of mAKAP-associated RyR, mAKAP was immunoprecipitated from purified nuclei, and RyR was detected in anti-mAKAP-specific immunoprecipitates by immunoblotting (Fig. 4C, lane 2).

Recombinant PP2A, RyR and mAKAP interaction

Given the association of RyR and mAKAP in cells, we were interested in studying their interaction at the molecular level using a pull-down assay (Fig. 5). Mutations have been found in the cytosolic, N-terminal domain of human type II RyR in

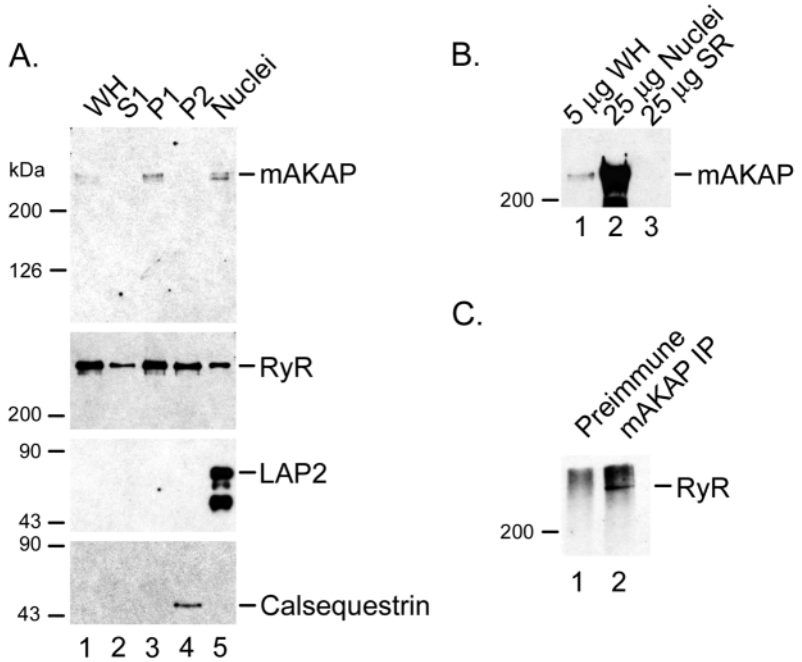

Fig. 4. mAKAP-RyR complex is specific to the nucleus. (A) Adult rat hearts were fractionated for comparison of the constituents of different membrane compartments. Whole heart homogenate (WH, lane 1) was centrifuged at 3800 ***g***. The resulting pellet (P1, lane 3) contained myofibrils, mitochondria and nuclei. Nuclei (lane 5) were purified from P1 by 2.4 M sucrose step gradient centrifugation at 50,000 ***g*** and repeat low-speed centrifugation. The 3800 ***g*** supernatant fraction (S1, lane 2) was clarified at 10,000 ***g***, before centrifugation at 100,000 ***g***. The 100,000 ***g*** pellet (P2, lane 4) contained sarcoplasmic reticulum, Golgi apparatus and plasma membrane. 5 µg protein for each fraction was subjected to SDS-PAGE and immunoblotting with purified mAKAP antibody and monoclonal RyR antibody. Antibodies for LAP-2, a nuclear matrix protein, and calsequestrin, a SR calcium-binding protein, served as indicators for the quality of the fractionations. (B) Comparison with pure SR. 5 µg whole heart homogenate (WH, lane 1), 25 µg purified nuclei (lane 2), and 25 µg purified sarcoplasmic reticulum (SR, lane 3) were subject to immunoblotting with anti-mAKAP VO54 antibody. SR fraction was prepared from P2 fraction by sucrose gradient centrifugation. (C) mAKAP and RyR are still associated after purification of nuclei. 75 µg of purified heart nuclei were treated as starting material in immunoprecipitation experiments with control preimmune serum (lane 1) or specific mAKAP VO54 antiserum (lane 2), and RyR was detected in immunoprecipitates by immunoblotting as performed in Fig. 2. As assessed by light microscopy, the nuclei used in these experiments remained intact throughout their purification (not shown). The migration of the respective protein and molecular weight markers are indicated for each panel. *n*=3 for the experiments represented in each panel.

patients with arrhythmogenic right ventricular dysplasia (ARVD), a cardiac disorder often presenting as sudden death (Tiso et al., 2001). Because the human mAKAP gene is linked to another genetic locus for ARVD (Kapiloff et al., 1999), we speculated that one of the RyR mutations might lie within the RyR domain responsible for mAKAP association, thereby facilitating the mapping analysis of this very large protein. Mutations have been found at RyR aa residues Arg176, Leu433, Asn2386 and Thr2504 in ARVD (Tiso et al., 2001), and in another genetic disorder, catecholaminergic polymorphic ventricular tachycardia (CPVT), at RyR aa residues Ser2246, Arg2474 and Arg4497 (Priori et al., 2001). GST-fusion proteins including large regions of RyR encompassing these mutations were produced in bacteria (Fig. 5A). Full-length mAKAP was expressed by infection with adenovirus vectors in HEK293 and COS-7 cells. Whole cell extracts containing mAKAP were then incubated with glutathione beads previously absorbed with GST-fusion proteins or GST alone (Fig. 5B). A GST-fusion protein containing RyR aa residues 1-568 specifically mediated the precipitation of mAKAP protein (lane 1). By contrast, GST-fusion proteins containing RyR aa residues 2080-2609 or 4332-4663 (lanes 2,3) and GST-alone (lane 4) were unable to bind mAKAP and serve as controls for these experiments. Similar amounts of GST fusion proteins were used in all experiments (data not shown). In parallel assays, it was apparent that a GST-PP2A A-subunit fusion protein was also effective at pulling down mAKAP (Fig. 5C). These data serve

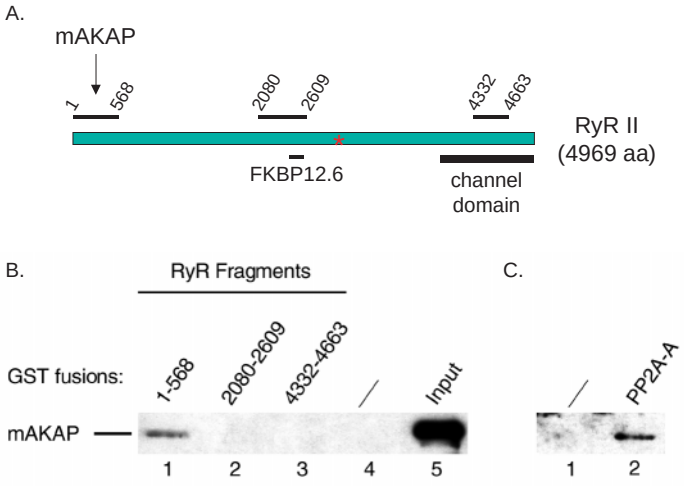

Fig. 5. mAKAP is precipitated by an N-terminal fragment of RyR type II and the A-subunit of PP2A. mAKAP was expressed in HEK293 cells, and whole cell extracts were subjected to GST-pull down assay. Polypeptides fused to GST were full-length PP2A A-subunit and RyR aa residues 1-568, 2080-2609 and 4332-4663. (A) A schematic diagram showing the size of each RyR fragment and its location within the primary structure of the protein. For reference, the binding site for FKBP12.6 and the ion channel domain on type II RyR are indicated (see 'Discussion'). Type II RyR is thought to be multiply phosphorylated by plural protein kinases, but only one site (Ser2809, denoted by asterisk) has been mapped, and by which kinase this residue is phosphorylated remains unclear (MacKrill, 1999; Marx et al., 2000). (B) Proteins pulled down by GST-RyR fusion proteins were subjected to SDS-PAGE, and mAKAP was detected by immunoblotting with purified VO56 antibody. Panel C. Identical experiment performed with GST-PP2A A-subunit and GST alone. The migration of mAKAP is indicated. The panels are representative of three individual experiments.

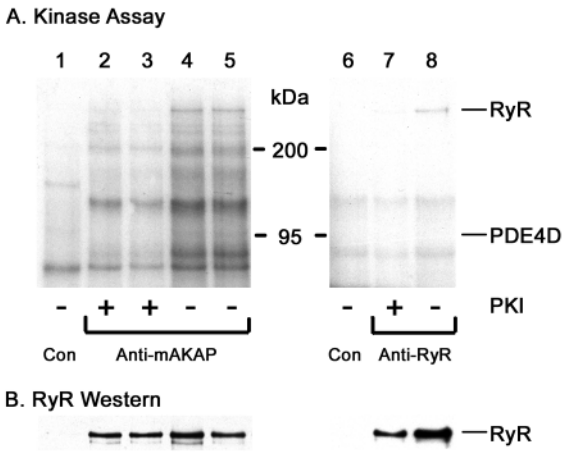

Fig. 6. Ryanodine receptor (RyR) is phosphorylated by PKA in both anti-mAKAP and anti-RyR immunoprecipitates from whole heart. (A) Assay of endogenous protein kinase activity. Solubilized whole heart extracts were prepared as in Fig. 2, and protein complex was immunoprecipitated with mAKAP antiserum (lanes 1-5) or anti-RyR specific antibody (lanes 7-8). Immune complexes were incubated with [γ-^{32}P]ATP and cAMP in the absence (lanes 1,4,5,6,8) or presence (lanes 2,3,7) of the specific PKA inhibitor PKI. Controls (Con) included immunoprecipitation in the presence of excess mAKAP C-terminal antigen (lane 1) or with mouse IgG (lane 6). Lanes 2-5 represent duplicate reactions. Phosphorylation was detected by autoradiography after protein fractionation by SDS-PAGE and transfer to nitrocellulose. Bands containing PDE4D and RyR proteins were identified by subsequent immunodetection with the appropriate antibodies and are indicated respectively, although phosphorylated-PDE4D is only faintly detectable in the exposure shown. Molecular weights are as indicated. (B) RyR immunoblot. Equal loading was verified by immunodetection with anti-RyR antibodies. Panels are representative of three individual experiments.

to map the mAKAP-interaction site on RyR type II to the N-terminal tenth of the RyR protein and to support the association of mAKAP with RyR and the core PP2A heterodimer in cells, in agreement with their co-immunoprecipitation with mAKAP antiserum from heart extracts.

RyR is a mAKAP-associated PKA substrate

Having shown that mAKAP, PKA and RyR associate in a NE-bound complex and because the RyR is regulated by PKA phosphorylation (MacKrill, 1999), we were interested in whether mAKAP-bound RyR may be a substrate for the associated PKA. RyR was immunoprecipitated from whole heart homogenate using anti-mAKAP VO54 antiserum or anti-RyR monoclonal antibody (Fig. 6). Immunoprecipitated complexes were assayed for endogenous PKA activity by contrasting incorporation of [^{32}P]phosphate in the absence and presence of the specific PKA inhibitor PKI (Fig. 6A). Equal loading was verified and the RyR band was identified by immunoblotting the same nitrocellulose filter with anti-RyR antibody (Fig. 6B). RyR was consistently radiolabelled two- to fourfold more intensely in the absence of PKI, regardless of antibody used for immunoprecipitation (Fig. 6A, compare lanes 2-5 and 7,8). Negative controls included absorption of VO54 antiserum with excess mAKAP antigen prior to immunoprecipitation (Fig. 6, lane 1) and immunoprecipitation with preimmune antiserum (not shown) and non-immune IgG (Fig. 6, lane 6). It is important to note that, in these assays, anti-mAKAP immunoprecipitates should represent only a NE pool of RyR, while anti-RyR immunoprecipitates should include RyR from all compartments, of which the SR is by far the largest. The phospho-protein profile for anti-mAKAP immunoprecipitates (Fig. 6A, lanes 4,5) is different from that for RyR immunoprecipitates (Fig. 6A, lane 8), probably an indication of the different constituency of NE and SR RyR pools.

DISCUSSION

Ca^{2+} and cAMP are critical second messengers in cardiac signal transduction. Ca^{2+} fluxes directly control the contractile cycle and are at the center of excitation-coupling and regulation of cellular hypertrophy (Frey et al., 2000; Katz, 1996). cAMP serves to modulate inotropy, chronotropy and lusitropy, mediating the effects of β-adrenergic stimuli by inducing PKA phosphorylation of contractile proteins, ion channels, enzymes of intermediary metabolism and other regulatory proteins (Walsh and Van Patten, 1994). Crosstalk between cAMP- and Ca^{2+}-dependent pathways is essential for normal cardiac physiology and occurs by multiple, often localized mechanisms (Colledge and Scott, 1999). For example, effective AKAP-mediated targeting of PKA is necessary in ventriculocytes for the β-adrenergic-mediated phosphorylation of troponin I and myosin basic protein C, two regulators of myofibrillar Ca^{2+} sensitivity (Fink et al., 2001). By association with the RyR Ca^{2+} release channel, the PKA-mAKAP complex may permit the integration of cAMP and Ca^{2+} signals at the cardiomyocyte NE.

mAKAP staining has been shown to be limited to the NE of cardiomyocytes in frozen sections of rat cardiac tissue and in cultured myocytes using two independent, affinity-purified anti-mAKAP antibodies (Dodge et al., 2001; Kapiloff et al., 1999). We now extend those studies by showing that mAKAP is localized at the cardiomyocyte NE of paraffin-embedded human ventricular tissue (Fig. 1). Our results are strengthened by data that GFP fused to mAKAP is directed solely to the NE of transfected rat neonatal ventriculocytes and that two adjacent domains of mAKAP (aa residues 772-915 and 915-1065) containing spectrin-like repeats can also direct GFP to the NE (Kapiloff et al., 1999). These results differ from two reports that have found mAKAP in multiple myocyte compartments (Marx et al., 2000; Yang et al., 1998). We cannot exclude the possibility that mAKAP is targeted to other intracellular sites at levels beneath the threshold of detection in these experiments.

To investigate the function of the mAKAP targeting of a select pool of PKA (Fig. 3E-H) and PDE4D3 (Dodge et al., 2001) to the nuclear envelope, we have begun to define the other components of the mAKAP complex. Co-immunoprecipitation using anti-mAKAP antibodies revealed the association of mAKAP with PP2A and RyR, but not with a variety of other signaling proteins including calcineurin, PP1, SERCA2A and PLB (Fig. 2). PP2A is a heterotrimeric protein phosphatase implicated in the de-phosphorylation of multiple signaling enzymes, including protein kinase C, casein kinase, Ca^{2+}/calmodulin-dependent protein kinases, mitogen activated protein kinases, cyclin-dependent protein kinases, and both PKA and RyR (Chu et al., 1990; Millward et al., 1999). In the heart, PP2A may be involved in contractile protein and ion-

channel regulation as well. Interestingly, transgenic mice expressing a dominant negative form of PP2A A-subunit exhibit cardiac hypertrophy and die from dilated cardiomyopathy (Brewis et al., 2000). The regulation of PP2A is poorly understood and specificity is thought to be, in part, secondary to targeting by different B-subunits (Millward et al., 1999). PP2A C-subunit was detected in anti-mAKAP immunoprecipitates using heart extract (Fig. 2), and mAKAP expressed in HEK293 cells was precipitated with GST-A-subunit (Fig. 5C). It remains to be determined whether the mAKAP-PP2A interaction is indirect or whether mAKAP serves as a B-subunit to target the PP2A core heterodimer to the NE. PP2A has been found to associate with RyR aa residues 1451-1768, but whether this interaction is direct is not known (Marx et al., 2000). Given the extremely low levels of RyR in HEK293 cells (Querfurth et al., 1998), it is not likely that mAKAP bound PP2A through RyR in the pull-down experiments. Future studies will address the mechanism of binding and the role of PP2A in the mAKAP complex.

Having detected RyR in mAKAP immunoprecipitates, we investigated whether there was partial co-distribution of RyR and mAKAP at the cardiomyocyte NE. Immunocytochemistry of rat neonatal ventriculocytes revealed that RyR is principally an SR ion channel (Fig. 3B), consistent with its well-understood role in excitation-coupling (Franzini-Armstrong and Protasi, 1997). Importantly, there was a significant pool of RyR also at the NE, overlapping in distribution with mAKAP (Fig. 3A-D). These results were followed by experiments involving fractionation of adult rat heart tissue (Fig. 4). RyR was found in both microsomal and nuclear fractions (Fig. 4A), while mAKAP was present in nuclear, but not SR fractions (Fig. 4B). Confirming the presence of RyR-mAKAP complex at the NE, RyR and mAKAP were co-immunoprecipitated from purified nuclei (Fig. 4C). mAKAP-RyR co-immunoprecipitation has been independently demonstrated in a manuscript concerning the SR RyR macromolecular complex (Marx et al., 2000). One strength of the experiments presented here is that, in order to distinguish between pools of RyR, specific precaution that has not traditionally been warranted in RyR investigations was employed during homogenization to prevent nuclear disruption and mixing of NE and SR membranes (Meissner, 1974; Tata, 1974). The results of experiments involving immunohistochemistry and tissue fractionation lead us to conclude that mAKAP associates predominantly with a NE pool of RyR, and not significantly with RyR present at the SR.

Only recently has it become appreciated that a pool of RyR is resident on the NE (Bootman et al., 2000). Ca^{2+} currents sensitive to cADPr and anti-RyR staining have been detected on the NE of isolated nuclei (Adebanjo et al., 1999; Gerasimenko et al., 1995). Thus far, no molecular differences have been discerned between SR and NE RyRs. Type I RyR has been found at the NE of osteoclasts, cells that apparently do not contain type II or type III RyR (Adebanjo et al., 2000). mRNA for all three types of RyR have been detected in heart (Franzini-Armstrong and Protasi, 1997). Because the RyR antibody used in this study is selective, but not exclusive for type II RyR, and because the cytoplasmic domains of the RyR forms are highly similar, we cannot exclude the possibility that cardiac NE RyR is type I or III, rather than type II, the predominant form in the heart.

Our data suggest that an important difference between SR and NE RyR is the selective association of NE RyR with the mAKAP complex (Fig. 3). By RIIα-overlay assay, there is a highly abundant AKAP of about 140 kDa M_r in heart that is the major RIIα-binding protein in SR preparations (data not shown). This putative SR-AKAP may be associated with SR RyR and may be important to excitation-coupling. More investigation is required to discern what other signaling components are associated with NE mAKAP-RyR complex, including those represented by the multiple phosphorylated bands found for mAKAP complex (Fig. 6, lanes 4,5). Although calcineurin and PP1 have been reported to be associated with RyR (Cameron et al., 1995; Marx et al., 2000), we detected neither in mAKAP immunoprecipitates (data not shown), reflecting the difficulty of higher order complex co-immunoprecipitation or, potentially, the different constituency of SR and NE RyR complexes. In addition, given the different concentrations of mAKAP and RyR in the microsomal (P2) and nuclear fractions (Fig. 4) and the very different staining patterns for mAKAP and RyR in cells (Fig. 3), a molecularly distinct subset of RyR or a protein other than RyR that is yet unidentified must serve as the NE anchor for mAKAP.

The RyR is a large ion channel with multiple functional domains (Fig. 5A). As much as the C-terminal fifth of the protein contributes to the ion channel core, while the extensive N-terminal ‘foot’ domain and the C-terminus of RyR are cytosolic and can be involved in protein-protein interactions (Franzini-Armstrong and Protasi, 1997). Although the characterization of the interaction with most RyR modifiers remains incomplete or controversial (MacKrill, 1999), the site for FKBP12.6 has been mapped to within RyR aa residues 2361-2496 (Marx et al., 2000), near or overlapping where RyR type II mutations have been found in human disease (see ‘Results’). We have begun to define the mAKAP binding domain on RyR using GST-fusion proteins (Fig. 5A,B). A GST-fusion protein containing the N-terminal 568 aa residues of type II RyR can specifically mediate mAKAP precipitation, in contrast to two other large regions of the RyR or GST alone. This N-terminal region is also the site of mutations found in patients with ARVD (Tiso et al., 2001). It remains to be determined whether the RyR-mAKAP interaction is affected in ARVD or other forms of cardiomyopathy.

The RyR has been well studied as a mediator of calcium-induced calcium release. It is tightly regulated by the endogenous ligand cyclic ADP ribose (cADPr), by Ca^{2+} itself, by multiple protein-protein interactions, and by protein kinases including PKA and Ca^{2+}/calmodulin-dependent protein kinase (MacKrill, 1999). By increasing the RyR sensitivity to Ca^{2+} and the rate of channel closure, PKA phosphorylation of the RyR can contribute in the heart to higher amplitude, faster cycling pulses of intracellular Ca^{2+} during states of increased inotropy and chronotropy (Valdivia et al., 1995). PKA-dependent phosphorylation has also been associated with decreased inhibition of the RyR by the constitutively high intracellular levels of magnesium ion (Hain et al., 1995) and with the dissociation of FKBP12.6 from the cardiac RyR (Marx et al., 2000). NE RyR is probably regulated in a similar manner by mAKAP-sequestered PKA in cells, for RyR is phosphorylated by endogenous PKA in isolated native mAKAP complex (Fig. 6).

Current ion channel theory holds that elementary, transient

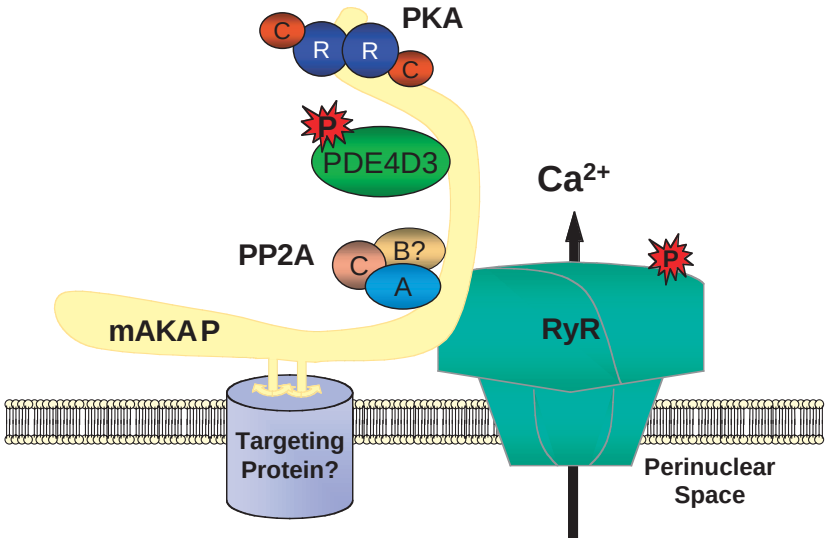

Fig. 7. Model of mAKAP complex. mAKAP associates with PKA, PDE4D3, PP2A and RyR at the cardiomyocyte NE. mAKAP is targeted to the NE by two spectrin-like repeat-containing domains (aa residues 772-915 and 915-1065) and binds PKA via an amphipathic helix at aa residues 2055-2072. mAKAP is associated with the N-terminal 568 aa of the RyR. PKA can phosphorylate RyR and PDE4D3, leading to increased ion channel and phosphodiesterase activity (a negative feedback loop; Dodge et al., 2001). PP2A may serve to reverse these phosphorylation events.

Ca^{2+} currents through individual channels called 'puffs' and 'sparks' can be locally important and may, if frequent, give rise to generalized changes in cellular Ca^{2+} levels (Bootman et al., 2000). In situ Ca^{2+} imaging has been used to demonstrate that nucleoplasmic Ca^{2+} levels in cultured cardiomyocytes and isolated nuclei can be affected autonomously by NE RyR channels (Abrenica and Gilchrist, 2000; Adebanjo et al., 1999; Adebanjo et al., 2000). Ca^{2+}-currents derived from the NE are apparently involved in the regulation of nuclear import (Jans and Hubner, 1996) and Ca^{2+}/calmodulin-dependent protein kinase-regulated cardiac gene transcription (Chawla et al., 1998; Heist and Schulman, 1998). The mAKAP complex that includes PKA, PDE4D3, PP2A and RyR is strategically located to modulate Ca^{2+}-regulated nuclear events (Fig. 7). cAMP may increase perinuclear Ca^{2+} fluxes by PKA phosphorylation of the RyR, in a manner tightly controlled by the PKA-activated PDE4D3 and PP2A-mediated dephosphorylation. Alternatively, because the RyR is Ca^{2+}-sensitive, it is possible the RyR serves as a sensor for ambient Ca^{2+} levels, controlling signal transduction to the nucleus though the other mAKAP-bound signaling enzymes and participating in a complex web of phosphorylation-induced positive and negative feedback loops.

Due to its location at the NE, the mAKAP-RyR complex should serve to integrate cAMP and Ca^{2+} signals in the regulation of nuclear processes. Changes in cAMP- and Ca^{2+}-dependent signal transduction are part of the cardiac response to stress. β-adrenergic pathways (Post et al., 1999), MAP-kinase signaling (Sugden and Clerk, 1998), and Ca^{2+}-dependent pathways involving calmodulin-dependent kinases, calcineurin and MEF2 (Frey et al., 2000) contribute to the changes in gene expression and cytoskeleton that occur in cardiac hypertrophy both during exercise and in disease states. Mutations in type II RyR are found in two distinct human diseases, CPVT and ARVD (Priori et al., 2001; Tiso et al., 2001). Although patients with both disorders have a high incidence of sudden death, ARVD patients suffer from a distinct group of arrhythmias. ARVD is characterized by degeneration and fibro-fatty infiltration of particularly the right ventricular myocardium, mostly likely due to inappropriate apoptosis (Runge et al., 2000). Abnormal RyR function has been associated with the induction of apoptosis (Hajnoczky et al., 2000). The human gene for mAKAP is also linked to a locus for ARVD (Severini et al., 1996). Our current investigations into whether mutations in mAKAP are found in ARVD and into the structure-function relationships of the mAKAP-RyR complex should extend our understanding of cAMP and Ca^{2+} signaling and may provide new insights into the pathogenesis of ARVD, a poorly understood cause of human early mortality.

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