Identification of a 15-kDa cAMP-dependent Protein Kinase-anchoring Protein Associated with Skeletal Muscle L-type Calcium Channels*

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Voltage-dependent potentiation of skeletal muscle L-type calcium channels requires phosphorylation by cAMP-dependent protein kinase (PKA) that is localized by binding to a cAMP-dependent protein kinase-anchoring protein (AKAP). L-type calcium channels purified from rabbit skeletal muscle contain an endogenous co-purifying protein kinase activity that phosphorylates the α1 and β subunits of the channel. The co-purifying kinase also phosphorylates a known PKA peptide substrate, is stimulated by cAMP, and is inhibited by PKA inhibitor peptide-(5–24), indicating that it is PKA. PKA activity co-immunoprecipitates with the calcium channel, suggesting that the channel and the kinase are physically associated. Using biotinylated type II regulatory subunit of PKA (RII) as a probe, we have identified a 15-kDa RII-binding protein in purified calcium channel preparations, which we have designated AKAP-15. Anti-peptide antibodies directed against the α1 subunit of the calcium channel co-immunoprecipitate AKAP-15. Together, these findings demonstrate a physical link between PKA and the calcium channel and suggest that AKAP-15 may mediate their interaction.

The activation of PKA† following transient increases in intracellular levels of cAMP represents a fundamental mechanism for regulating protein function via phosphorylation (1, 2). A wide variety of proteins including enzymes, membrane receptors, ion channels, and transcription factors have been shown to be PKA substrates with activities reversibly modulated by phosphorylation and dephosphorylation. It is clear that despite its broad substrate specificity, PKA activity is highly selective in a physiological setting and that specific hormones, each capable of raising intracellular cAMP, represents a fundamental mechanism for regulating protein function via phosphorylation (1, 2).

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The abbreviations used are: PKA, cAMP-dependent protein kinase; RII, type II regulatory subunit of PKA; AKAP, cAMP-dependent protein kinase-anchoring protein; PAGE, polyacrylamide gel electrophoresis; MAP-2, microtubule-associated protein-2; Tricine, N-[2-hydroxy-1.1-bis(hydroxymethyl)ethyl]glycine; PKI-(5–24), PKA inhibitor peptide-(5–24); MOPS, 4-morpholino-propanesulfonic acid; TBS, Tris-buffered saline.

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Acid/kainate glutamate receptor ion channels in hippocampal neurons require PKA phosphorylation for the maintenance of inward currents (22), and the presence of peptides designed to disrupt RII-AKAP binding results in a diminishment of the current probably by displacing anchored PKA from the channel (23). The importance of PKA anchoring via AKAPs has also been established in the regulation of L-type calcium channels. In skeletal muscle transverse tubules, these channels initiate muscle contraction by directly interacting with ryanodine receptors to cause the release of calcium from the sarcoplasmic reticulum (24). The calcium entering directly through L-type calcium channels, on the other hand, is thought to be important in regulating the force of contraction. High frequency stimulation of muscle fibers causes potentiation of skeletal muscle calcium channel activity, an effect that may mediate the increased force of muscle contraction during tetanus (25). This frequency- and voltage-dependent potentiation of calcium channel activity has been shown to require phosphorylation by PKA since an inhibitor peptide of PKA blocks the effect (25). Recent electrophysiological studies have shown that this effect requires the anchoring of PKA near the channel. The introduction of an anchoring inhibitor peptide into skeletal muscle myotubes, presumably by displacing PKA from an endogenous AKAP (26). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor and L-type calcium channel therefore provide two examples demonstrating the physiological importance of PKA anchoring.

The electrophysiological evidence that PKA is anchored to the L-type calcium channel complements biochemical studies. It was demonstrated that an endogenous protein kinase activity co-purifies with the calcium channel through several chromatographic steps, suggesting that it might be physically associated with the channel (27). In the present study, we identify co-purifies with the calcium channel through several chromatographic steps. PKA anchoring therefore provides two examples demonstrating the physiological importance of PKA anchoring.

EXPERIMENTAL PROCEDURES

Materials—10–20% Tricine gels and molecular weight markers were obtained from Novex (San Diego, CA). cAMP, cAMP-agarose, protein A-Sepharose, and MAP-2 were purchased from Sigma. EZ™ sulfosuccinimidyl 6-(biotinamido)hexanoate, calpain I, and isopropyl-β-D-thiogalactopyranoside, horse serum, and chemiluminescent substrate (Supersignal™) were from Pierce. [γ-32P]ATP (3000 Ci/mmol) was obtained from DuPont NEN. SDS-PAGE gels, Tris-HCl (pH 6.8), 10% glycerol, 10 mM dithiothreitol, and 2% SDS were from Bio-Rad. 4-(aminoethyl)benzenesulfonyl fluoride (1 mM), antipain (1.6 mM), and leupeptin (2 mM) were synthesized and purified in the University of Washington Molecular Pharmacology Protein Core Facility. DSDD peptide (RRRDDDS-LTYEKRFSSPHQSLLSIR), and SP44 (KYMKKLGSKKPQK) peptides were synthesized and purified in the University of Washington Chemical Laboratories, Inc. (Belmont, CA). HT-31, HT-31-P (18, 26), SP48 (N-terminal) and SP44 (C-terminal) peptides were synthesized and purified in the University of Washington Chemical Laboratories, Inc. (Belmont, CA). [γ-32P]ATP (3000 Ci/mmol) was obtained from DuPont NEN. SDPs were synthesized and purified in the University of Washington Molecular Pharmacology Protein Core Facility. DSDD peptide (RRRDDDS-D) was a gift from Ed Krebs (University of Washington).

Preparation of Skeletal Muscle Calcium Channels—Skeletal muscle calcium channels were purified as described by Curtis and Catterall (28) from skeletal muscle microsomes prepared according to Fernandez et al. (29). All buffers contained the following protease inhibitors: 4-(aminomethyl)benzenesulfonyl fluoride (1 mM), leupeptin (2 μM), pepstatin A (1 μM), antipain (1.6 μM), calpain inhibitor I (10 μg/ml), calpain inhibitor II (10 μg/ml), and o-phenanthroline (0.9 mM). The picomoles of purified calcium channel were estimated by assuming a molecular mass of 429 kDa for the channel complex.

Phosphorylation of the Calcium Channel and Synthetic Peptides by the Endogenous Kinase—Purified calcium channels were phosphorylated by incubation at 37 °C in 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 10 mM MgCl₂, 1 mM EGTA, and 0.15 μM [γ-32P]ATP (3000 Ci/mmol) for the times indicated. Calcium channel phosphorylation reactions were terminated by heating at 65 °C for 3 min in 50 mM Tris-HCl (pH 6.8), 10% glycerol, 10 mM dithiothreitol, and 2% SDS and subsequently analyzed by SDS-PAGE. In some cases, individual phosphoprotein bands were excised from the gels, and [32P] was quantified by liquid scintillation counting.

Purified synthetic peptides were phosphorylated by incubation at 37 °C in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, and 0.5 mM [γ-32P]ATP (80 μCi/mmol) for 10 min in the presence of 1–5 pmol of purified calcium channel. Phosphorylation reactions were terminated by acidification to 16% acetic acid, spotted on P-81 paper, washed with 0.1% phosphoric acid, and quantified by liquid scintillation counting.

Expression, Purification, and Biotinylation of RIIa—The expression plasmid pET11d containing the mouse RIIα cDNA (30) was transformed into Escherichia coli BL21(DE3) competent cells (Novagen). The RIIα vector was kindly provided by John Scott (Vollum Institute, Oregon Health Sciences University). 25-ml overnight cultures were grown in LB medium containing 100 μg/ml ampicillin (LBA) and used to inoculate 1 liter of LBA. Cells were then grown by shaking at 37 °C until the A₅₅₀ of the 1-liter culture reached 0.300. Expression of RIIa was subsequently induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and incubation was continued for an additional 2.5 h. Cells were pelleted by centrifugation at 2200 × g for 10 min and resuspended in 20 ml of 10 mM MOPS (pH 6.9), 100 mM NaCl, and 1 mM dithiothreitol containing aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (1 μM), o-phenanthroline (0.2 mg/ml), 4-(aminomethyl)benzenesulfonyl fluoride (1 mM), and benzamidine (15.7 μg/ml) (lysate buffer). Resuspended cells were lysed by mild sonication with a probe sonicator for 30 s on ice and centrifuged at 10,000 × g for 15 min. Pellets were discarded, and 10 μl of supernatant was added to the supernatants while stirring on ice to achieve 80% saturation. Stirring on ice was continued for 15 min to allow protein precipitation to reach equilibrium, and precipitated proteins were separated from soluble material by centrifugation at 10,000 × g for 30 min. Supernatants were discarded, and ammonium sulfate precipitates were resuspended in 20 ml of lysate buffer containing 10 μM isobutylmethylxanthine in addition to the protease inhibitors listed above. Resuspended material was mixed with 5 ml of cAMP-agarose and rotated for 16 h at 4 °C. Nonspecifically bound proteins were removed by washing the cAMP-agarose with 50 ml of lysate buffer, followed by washing with 50 ml of lysate buffer containing 1 mM NaCl and finally washing again with 50 ml of lysate buffer. Bound RII protein was eluted by incubating cAMP-agarose with 15 ml of lysate buffer containing 25 mM free cAMP for 30 min at room temperature. The eluate was concentrated by centrifugation in a Centriprep 30 concentrator (Amicon, Inc.) according to the manufacturer’s instructions.

Purified recombinant RIIa was biotinylated by dialyzing 0.5 ml of pure RII protein (0.5–1.5 mg/ml) against 2 liters of 50 mM sodium bicarbonate (pH 8.5) for 2–4 h. EZ™ sulfosuccinimidyl-6-(biotinamido)hexanoate was then added at a 10-fold molar ratio over protein and incubated on ice for 2 h. Excess unreacted biotin was removed by dialysis overnight against 10 mM Tris-HCl (pH 7.4) and 0.15 mM NaCl (TBS). Polyacrylamide Gel Electrophoresis, Immunoblotting, and RII Overlay—SDS-PAGE was carried out under reducing conditions on 10–20% polyacrylamide-Tris/Tricine gels. Electrophoretic transfer to nitrocellulose membranes was carried out in a Novex Xcell II apparatus for 2.5 h at 195 mA (constant current) with 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol (buffer H) as the transfer buffer. Unbound spots were blocked either for 30 min at room temperature or overnight at 4 °C with 5% (v/v) skim milk powder or 10% normal horse serum in TBS. Blots were blocked in 10% normal horse serum only prior to detection of RII, which involved the use of an anti-goat antibody that cross-reacted with proteins from milk, but not horse serum.

For AKAP detection, blocked membranes were washed three times for 10 min each with TBS containing 0.05% Tween 20 (TBS) and then incubated for 1 h with 5 mM RII-biotin with or without a 30-min room temperature preincubation with 0.4 μM HT-31 or HT-31-P peptide in TBS. Membranes were then washed three times for 10 min each with TBS and incubated for 30 min with 2 μg/ml horseradish peroxidase-linked avidin in TBS. Blots were washed three times for 10 min each with TBS, and reactive bands were visualized using the Pierce SuperSignal™ ECL detection system. For RII detection, nitrocellulose membranes blocked in horse serum were incubated for 1 h with a goat anti-RII antibody (kindly provided by John Scott, Vollum Institute, Oregon Health Sciences University), followed by a 30-min incubation with a horseradish peroxidase-linked anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc.). Detection was via ECL and TBS-T washes were included between steps as described above. Developed calcium channel was detected following a 1-h incubation with the rabbit anti-CP11 peptide antibody directed against the C-terminal region of the α1 subunit, followed by a 30-min incubation with horseradish peroxidase-linked protein A, ECL, and film exposure.

Co-immunoprecipitation of the Endogenous Kinase Activity and AKAP-15 with the Calcium Channel—Approximately 10 pmol of cal...
RESULTS

Purified Rabbit Skeletal Muscle Calcium Channels Contain Endogenous Kinase Activity—Incubation of purified calcium channels with \([\gamma^{32}\text{P}]\text{ATP}\) followed by SDS-PAGE and autoradiography as described under “Experimental Procedures” yielded three prominent phosphoprotein bands of 170, 65, and 60 kDa, with a less prominent band appearing at 90 kDa (Fig. 1A). Fig. 1A also shows that the phosphorylation of these proteins by the endogenous kinase increased over time. To identify the three phosphorylated proteins, we performed immunoblotting experiments on identical purified calcium channel preparations. The 170-kDa phosphoprotein seen in Fig. 1A displayed the same apparent mobility as the \(\alpha_1\) subunit of the calcium channel recognized by anti-CP11 antibody (Fig. 1B, first lane). The 65-kDa phosphoprotein was similarly identified as the \(\beta\) subunit of the calcium channel based on comparison with the migration of the \(\beta\) subunit as recognized by a \(\beta\) subunit-specific antibody (Fig. 1B, second lane). Finally, the 60-kDa phosphoprotein was identified as RII as its gel position was the same as that of the RII protein recognized by RII-specific antibodies (Fig. 1B, third lane). The faint band at \(-90\) kDa was not identified and may represent a degradation product of the calcium channel \(\alpha_1\) subunit.

Endogenous Kinase Activity Resembles PKA—The presence of RII in purified calcium channel preparations as well as the fact that the calcium channel is a known substrate for PKA (31) led us to test whether the endogenous kinase was in fact PKA. Fig. 2A demonstrates the effect of cAMP on the endogenous kinase activity as measured by phosphorylation of the calcium channel \(\alpha_1\) subunit. The endogenous kinase activity was stimulated following the addition of cAMP. This experiment was repeated five times, and in each case, cAMP stimulated the phosphorylation of the \(\alpha_1\) subunit. cAMP-dependent stimulation ranged from \(-1.5\) to 3-fold. We further established the identity of the kinase present in calcium channel preparations using specific kinase substrate and inhibitor peptides. Fig. 2B shows that the endogenous kinase phosphorylated the known PKA substrate SP48 (32) and that phosphorylation of this peptide was stimulated by the addition of cAMP. cAMP consistently \((n = 5)\) increased the phosphorylation of SP48, with the stimulation ranging from \(-2.5\) to \(-8\)-fold. In addition, PKI-(5–24) completely blocked the cAMP-stimulated kinase activity (Fig. 2B). PKI-(5–24), however, did not completely block all endogenous protein kinase activity. The endogenous kinase activity failed to phosphorylate peptides that are substrates for PKC and casein kinase II (Fig. 2B). Together, these data strongly suggest that PKA is co-purifying with the calcium channel.

PKA Activity Co-immunoprecipitates with the Calcium Channel—We performed co-immunoprecipitation experiments to test whether the co-purifying kinase is physically associated with the calcium channel. The channel was first immunoprecipitated from calcium channel preparations with the \(\alpha_1\) subunit-specific anti-CP11 antibody and then incubated under phosphorylating conditions with \([\gamma^{32}\text{P}]\text{ATP}\) as described under “Experimental Procedures.” Fig. 3A shows that following immunoprecipitation and addition of \([\gamma^{32}\text{P}]\text{ATP}\), both the \(\alpha_1\) and \(\beta\) subunits of the calcium channel were phosphorylated by a co-purifying kinase. The PKA substrate peptide SP48 was also phosphorylated by the immunoprecipitated calcium channel complex (Fig. 3B). When the immunoprecipitation was performed with a control antibody (IgG), phosphorylation of the peptide was not detected (Fig. 3B). Therefore, based on co-immunoprecipitation experiments, the calcium channel is physically associated with a co-purifying kinase.

Detection of AKAPs with RII-Biotin Overlay—Recombinant RII protein expressed, purified, and biotinylated as described...
under “Experimental Procedures” was employed in an RII overlay assay adapted from the procedure of Lohmann et al. (12). Pure MAP-2 protein resolved by SDS-PAGE and immobilized on nitrocellulose was probed with RII-biotin and visualized with horseradish peroxidase-linked avidin and ECL as described under “Experimental Procedures.” Fig. 6 shows the presence of AKAP-15 among precipitated proteins as detected by the RII-biotin overlay assay. Detection of AKAP-15 was prevented by preincubation of RII-biotin with HT-31 peptide (Fig. 6). Moreover, AKAP-15 was not detected when a control antibody (IgG) was employed in place of anti-CP11 antibody (Fig. 6). Co-immunoprecipitation of AKAP-15 with the calcium channel confirms its physical association with the calcium channel complex.

**DISCUSSION**

Skeletal muscle L-type calcium channel activity is enhanced by PKA phosphorylation (25, 26, 33); also, purified calcium channels reconstituted into phospholipid vesicles or bilayers display an increase in ion flux activity following phosphorylation by PKA (34, 35). The channel complex purified from skeletal muscle transverse tubule membranes consists of five subunits: the principal pore-forming α1 subunit and auxiliary β, γ, α2, and δ subunits (24). Both the α1 and β subunits are phosphorylated by PKA in vitro (31, 34, 36, 37). The α1 subunit is phosphorylated on multiple serine residues by PKA both in vitro and in intact cells in response to cAMP stimulation (33, 38, 39). While the precise sites responsible for physiological modulation of calcium channel activity by PKA phosphorylation have not been identified, it is likely that critical sites lie on the α1 and/or β subunit of the calcium channel.
In this study, we have identified an endogenous kinase activity that co-purifies with the calcium channel. We have further provided evidence that this co-purifying kinase is PKA and that it is physically associated with the channel complex. Using a novel RII-biotin overlay assay, we have detected an AKAP of ∼15 kDa that also co-purifies and co-immunoprecipitates with the calcium channel. Together, these results suggest a role for AKAP-15 in anchoring type II PKA to the calcium channel and thereby allowing discrete phosphorylation of the channel in response to cAMP.

Our results are consistent with previous physiological experiments demonstrating a close association between L-type calcium channels and PKA (26). Voltage-dependent phosphorylation by PKA increases the subsequent activity of the calcium channel (25). This voltage-dependent potentiation represents a measurable effect of PKA on calcium channels in intact cells and has been used to assess the requirement of kinase anchoring. Johnson et al. (26) showed that the PKA-derived anchoring inhibitor peptide (HT-31) that disrupts RII-AKAP interaction completely blocks voltage-dependent potentiation of calcium channels in skeletal muscle myotubes. Blockade of voltage-dependent potentiation by disruption of anchoring was overcome by the introduction of excess catalytic subunit of PKA into cells at concentrations high enough to obviate the requirement for specific localization of PKA near the calcium channel (26). The ability of HT-31 peptide to block potentiation of the calcium channel by PKA strongly suggests that PKA is anchored near the channel by an AKAP. Our experiments are consistent with this hypothesis and suggest that AKAP-15 mediates the anchoring of PKA near the calcium channel.

The kinase activity we detect in purified calcium channel preparations resembles PKA in several respects. Our initial observation shows that this activity phosphorylates three PKA substrates, the α1 and β subunits of the calcium channel as well as RII. The endogenous kinase activity is also stimulated by cAMP, and the stimulated activity is inhibited by PKI (5–24). We measured the phosphorylation of various substrate peptides by the endogenous kinase and found that the PKA substrate SP48 was phosphorylated, while SP44, DSDD, PKC, and casein kinase II substrates, respectively, were not. Furthermore, two-dimensional tryptic phosphopeptide maps of the skeletal muscle calcium channel α1 subunit phosphorylated by either exogenous PKA or the endogenous kinase were similar (data not shown). While we believe we have identified PKA, it is important to note that we were unable to completely block the endogenous kinase activity with PKI (5–24), indicating that there may also be another kinase in these preparations.

The endogenous kinase activity not only co-purifies with the calcium channel, but co-immunoprecipitates with it as well. This result indicates that the kinase and calcium channel are physically linked to each other and provided the impetus to examine purified calcium channel preparations for AKAPs. Various approaches to detect AKAPs have been described, each involving the use of purified RII protein in an overlay assay (40). In this study, we have introduced a variation of the RII overlay using biotinylated RII. This new assay, which involves detection of RII-biotin with peroxidase-conjugated avidin, has proven faster and easier than detection with antibodies or with radiolabeled RII. We validated the RII-biotin overlay by using it to detect the known AKAP MAP-2. Preincubation of RII-biotin with an AKAP-derived anchoring inhibitor peptide (HT-31) prevented MAP-2 recognition, indicating that this is a specific RII-AKAP interaction. In addition to its usefulness in detecting AKAPs, we anticipate that RII-biotin will serve as a useful affinity reagent for purification of AKAPs.

Using the RII-biotin overlay, we have identified a novel 15-kDa AKAP in purified calcium channel preparations that we believe mediates the association of PKA and the calcium channel. Detection of AKAP-15 is blocked by preincubation of RII-biotin with HT-31 peptide, consistent with it being an AKAP. Co-purification of AKAP-15 with the calcium channel suggests that these two proteins are physically associated. We confirmed this association by co-immunoprecipitating AKAP-15 using a calcium channel-specific anti-peptide antibody. AKAP-15 was not co-precipitated by a control antibody, and its detection was blocked by preincubation of RII-biotin with HT-31 peptide. AKAP-15 is the only RII-binding protein we detected in purified calcium channel preparations, making it an excellent candidate for mediating the role of attaching PKA to the calcium channel complex. Unlike many AKAPs, which remain poorly characterized with respect to their specific targets and functional importance, AKAP-15 possesses both a likely target (L-type calcium channels) and functional importance (voltage-dependent potentiation). Purification and characterization of this AKAP may allow determination of its role in anchoring PKA to the calcium channel.

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