Specific Phosphorylation of a Site in the Full-Length Form of the α1 Subunit of the Cardiac L-Type Calcium Channel by Adenosine 3′,5′-Cyclic Monophosphate-Dependent Protein Kinase†

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ABSTRACT: Voltage-gated L-type Ca^{2+} channels mediate Ca^{2+} entry into cells in response to membrane depolarization. Ca^{2+} entry through the cardiac Ca^{2+} channel determines the rate and force of contraction, and modulation of Ca^{2+} channel activity by β-adrenergic agents acting through adenosine 3′,5′-cyclic monophosphate- (cAMP)-dependent protein phosphorylation contributes to physiological regulation of cardiac function by the sympathetic nervous system. Immunoblotting experiments using site-directed anti-peptide antibodies against different peptide segments indicate that the α1 subunit of the cardiac L-type Ca^{2+} channel exists in two size forms with apparent molecular masses of 240 and 210 kDa, which we call $\alpha1_{242}$ and $\alpha1_{210}$. $\alpha1_{242}$ corresponds to the full-length cardiac α1 subunit predicted from its cDNA sequence, while $\alpha1_{210}$ is truncated at its COOH terminus. Only $\alpha1_{242}$ is phosphorylated *in vitro* by cAMP-dependent protein kinase. Protein microsequencing and peptide mapping of wild-type and mutant fusion proteins show that this phosphorylation occurs at serine 1928 near the COOH terminus. Phosphorylation of this residue can be detected by phosphospecific antibodies raised against the corresponding phosphopeptide. Experiments with these antibodies show that $\alpha1_{242}$ is phosphorylated in intact cells expressing the cardiac α1 subunit in response to increased intracellular levels of cAMP. These results identify serine 1928 on the α1 subunit as a possible site of regulation by cAMP-dependent phosphorylation.

L-type voltage-gated Ca^{2+} channels in cardiac cells mediate the Ca^{2+} current which is responsible for the plateau phase of the action potential and for the Ca^{2+} entry that initiates contraction. Modulation of this current by neurotransmitters and hormones regulates the beat rate and force of contraction. β-Adrenergic stimulation enhances the Ca^{2+} current by stimulating the production of cAMP by adenylate cyclase and consequently activating phosphorylation of the L-type Ca^{2+} channel by cA-PK[1] (Reuter, 1983; Tsien et al., 1986; Trautwein & Heschler, 1990; Pelzer et al., 1990). However, the molecular mechanism of this physiologically important regulation of cardiac Ca^{2+} channel activity is unknown.

Ca^{2+} channels are minor proteins in most cell types including the heart. In contrast, skeletal muscle transverse tubule membranes contain a high density of L-type Ca^{2+} channels whose structure has been thoroughly characterized [reviewed in Catterall et al. (1988), Campbell et al. (1988), and Isom et al. (1994)]. The α1 subunit has a predicted size of 212 kDa, contains four internally homologous domains which are predicted to have six transmembrane helices (Tanabe et al., 1987; Figure 1) and is able to function autonomously as a voltage-gated Ca^{2+} channel when expressed in L-cells (Perez-Reyes et al., 1989). This subunit also binds Ca^{2+} antagonist drugs including the dihydropyridines (DHP). The α1 subunit and intracellular β subunit of 55 kDa are phosphorylated by multiple protein kinases which modulate channel activity (Curtis & Catterall, 1985; Flockerzi et al., 1986; Röhrkasten et al., 1988; O'Callahan & Hosey, 1988). The channel complex also contains a glycosylated α2 subunit of 143 kDa, which is disulfide-bonded to a glycosylated δ subunit of 24−29 kDa, and a glycosylated γ subunit of 30 kDa (Catterall et al., 1988; Campbell et al., 1988; Isom et al., 1994). Skeletal muscle Ca^{2+} channels are regulated by cAMP-dependent phospho-

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[1] Abbreviations: BSA, bovine serum albumin; cA-PK, adenosine 3′,5′-cyclic monophosphate-dependent protein kinase; CCAR3217, a Chinese hamster ovary cell line expressing the α1 subunit of the cardiac Ca^{2+} channel in the pCARD1 vector; CFH1−wt and CFH1−S1928A, glutathione S-transferase GST fusion proteins corresponding to residues 1895−1947 of the α1 subunit of cardiac Ca^{2+} channels with wild-type (wt) or mutant (S1928A) sequence; CH, cardiac calcium channel synthetic α1 subunit peptide; CHO, Chinese hamster ovary; CP, skeletal muscle calcium channel α1 subunit synthetic peptide; CR2, fusion protein corresponding to COOH-terminal 549 amino acid residues of the α1 subunit of cardiac calcium channels; DCl-cBIMPS, 5,6-dichloro 1-(β-D-ribofuranosyl)benzimidazole 3′,5′-cyclic monophosphorothioate, S_p isomer; DHP, dihydropyridine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; ELISA, enzyme-linked immunsorbent assay; GST, glutathione S-transferase; PAS, protein A−Sepharose; PCR, polymerase chain reaction; PEG, poly(ethylene glycol); TPCK, tosylamido-2-phenylethyl chloromethyl ketone; pCARD1, expression plasmid encoding the α1 subunit of the cardiac calcium channel; WGA, wheat germ agglutinin.

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rylation in skeletal muscle cells (Arreola et al., 1987; Schmid et al., 1985; Sculptoreanu et al., 1993), and incorporation of purified skeletal muscle Ca^{2+} channels into phospholipid vesicles or planar bilayers results in Ca^{2+} flux which is increased by cAMP-dependent phosphorylation (Flockerzi et al., 1986; Nunoki et al., 1989; Hymel et al., 1988; Mundina-Weilenmann et al., 1991). Two size forms of the α1 subunit are present in purified skeletal muscle Ca^{2+} channel preparations, skeletal muscle T-tubule membranes, and intact rat skeletal muscle cells in culture (De Jongh et al., 1989; Lai et al., 1990). The mass of the larger form, termed $\alpha1_{212}$, is approximately 210 kDa, in agreement with the size predicted for the α1 subunit from the cDNA clone (Tanabe et al., 1987). The smaller form accounts for more than 90% of the protein and has a molecular mass of 190 kDa due to a truncation between residues 1685 and 1699 in the COOH terminus (De Jongh et al., 1991). Serine residues specific to the COOH terminus of $\alpha1_{212}$ are rapidly phosphorylated by cAMP-dependent protein kinase (cA-PK) (Rotman et al., 1992, 1995).

The biochemical properties of cardiac Ca^{2+} channels are not as well-defined. A 165-kDa peptide from guinea pig heart membranes was photolabeled with the DHP [^{3}H]-azidopine (Ferry et al., 1987), while 185−195-kDa DHP-binding proteins were reported in preparations from chick (Chang & Hosey, 1988; Yoshida et al., 1990), rabbit (Tuana & Murphy, 1990), porcine (Tuana & Murphy, 1990; Haase et al., 1991; Kuniyasu et al., 1992), and bovine (Schneider & Hofmann, 1988) heart. These proteins are much smaller than the predicted size (242 kDa) of the α1 subunit of the rabbit cardiac L-type Ca^{2+} channel deduced from its cDNA sequence. Although cAMP-dependent phosphorylation of partially purified cardiac Ca^{2+} channel preparations containing α1 subunits of 165−195 kDa could not be demonstrated (Chang & Hosey, 1988; Schneider & Hofmann, 1988; Yoshida et al., 1990), Yoshida et al. (1992) reported phosphorylation of a 250-kDa α1 subunit form in cardiac microsomes and in Chinese hamster ovary (CHO) cells transfected with the cardiac α1 subunit cDNA. Stimulation of cA-PK in these transfected cells increased Ca^{2+} channel activity up to 2-fold (Yoshida et al., 1992), and a large (up to 20-fold) voltage-dependent potentiation of the Ca^{2+} channel activity was observed following dialysis with solutions containing cA-PK (Sculptoreanu et al., 1993). In the experiments described here, we show that cardiac sarcolemmal membranes contain both full-length and truncated forms of the α1 subunit, demonstrate that these two forms differ in their COOH-terminal domains, and identify a single site of cAMP-dependent phosphorylation in the COOH-terminal tail of the full-length cardiac α1 subunit *in vitro* and in intact cells which may be critical for regulation of the cardiac L-type Ca^{2+} channel.

EXPERIMENTAL PROCEDURES

Materials. Catalytic subunit of cA-PK was purified from bovine heart according to Kaczmarek et al. (1980). [^{3}H]-PN200−110 (70.9 Ci/mmol) and [γ-^{32}P]-ATP (3000 Ci/mmol) were purchased from Du Pont (Wilmington, DE), and digitonin was from Gallard-Schlesinger (Carle Place, NY). Wheat germ agglutinin (WGA)−Sepharose was from Pharmacia (Piscataway, NJ), and heparin−agarose and protein A−Sepharose were from Sigma (St. Louis, MO). Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)−trypsin was purchased from Worthington, and sequencing-grade modified porcine trypsin was obtained from Promega (Madison, WI).

Preparation of Skeletal Muscle Ca^{2+} Channels. Skeletal muscle Ca^{2+} channels were purified by chromatography on WGA−Sepharose and DEAE-cellulose (Curtis & Catterall, 1984) from skeletal muscle microsomes prepared according to Fernandez et al. (1980). All buffers contained the following protease inhibitors: phenylmethanesulfonyl fluoride (0.1 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 Ca^{2+} channel were estimated by assuming a molecular mass of 429 kDa for the channel complex.

Preparation of Cardiac Ca^{2+} Channels. Membrane fractions enriched in cardiac Ca^{2+} channels were prepared from rabbit heart ventricle muscle essentially as described by Murphy and Tuana (1990). The following protease inhibitors were included in all steps of cardiac Ca^{2+} channel isolation and purification: aprotinin (10 μg/mL), leupeptin (10 μg/mL), pepstatin A (1 μM), *o*-phenanthroline (0.2 mg/mL), phenylmethanesulfonyl fluoride (0.1 mM), benzamidine (15.7 μg/mL), calpain inhibitor I (10 μg/mL), calpain inhibitor II (10 μg/mL), and soybean trypsin inhibitor (10 μg/mL). Membrane fractions were assayed for DHP binding as described by Glossmann and Ferry (1985). Membrane protein (0.05−1.0 mg) was incubated with 5 nM [^{3}H]-PN200−110 in 10 mM Tris-HCl, pH 7.4, containing 1.5 mM $CaCl_2$ (buffer A) in a final volume of 0.5 mL for 90 min at room temperature in the dark. Bound and free ligand were separated on GF/C filters, and filters were washed three times with 3 mL of buffer A before liquid scintillation counting. Specific binding was defined as the component of total binding that was displaced by 5 μM unlabeled PN200−110.

Ca^{2+} channels were solubilized from cardiac membranes in 1% (w/v) digitonin with a 5:1 (w/w) detergent to protein ratio in 20 mM Tris-HCl and 150 mM NaCl, pH 7.4, at 4 °C for 30 min. Insoluble material was removed by centrifugation at 120000*g* for 30 min and the soluble fraction was equilibrated with 1.0 mL of WGA−Sepharose at 4 °C for 30 min. The Sepharose was packed into a column and washed with 50 mM Tris-HCl, 25 mM NaCl, and 0.1% (w/v) digitonin, pH 7.4 (buffer B). Adsorbed glycoproteins were eluted with 100 mM *N*-acetylglucosamine in buffer B and stored in liquid nitrogen until used. For immunoprecipitation experiments, Ca^{2+} channels were precipitated directly from the fraction eluted from the WGA−Sepharose column.

For immunoblotting experiments, cardiac Ca^{2+} channels were purified on WGA−Sepharose as outlined above and then adsorbed to heparin agarose in 20 mM Tris-HCl, 150 mM NaCl, and 0.1% (w/v) digitonin, pH 7.4 (buffer C) at 4 °C for 16 h. The agarose was pelleted by centrifugation and washed three times with buffer C and once with buffer C without digitonin. The adsorbed material was released by incubation with SDS−PAGE sample buffer at 70 °C for 15 min and loaded on a single lane of a polyacrylamide gel as outlined below.

Preparation of Antibodies. Synthetic peptides were synthesized by the solid-phase method (Merrifield, 1963) with NH_2-terminal Lys + Tyr and residues 1382−1400 [CP-(1382−1400)], 1419−1437 [CP-(1419-1437)], or 1692−1707 [CP-(1692−1707)] of the skeletal muscle Ca^{2+} channel

α1 subunit primary sequence or with residues 2051−2066 [CH-(2051−2066)], 2155−2171 [CH-(2155−2171)], or 1923−1932 [CH-(1923−1932)] of the cardiac α1 subunit primary sequence. Peptides were purified by reversed-phase HPLC on a Waters DeltaPak C18 column (25 × 300 mm, 15 μm, 300 Å) and their identities were confirmed by mass spectrometry. CH-(1923−1932) was phosphorylated in the presence of purified cA-PK and the resulting phosphopeptide ([CH-(1923−1932)-P]) was purified by reverse-phase HPLC. These peptide sequences are located within the COOH-terminal tail of the cardiac α1 subunit (see Figure 1 below).

Antisera against the peptides were prepared as outlined previously (Gordon et al., 1987). The anti-peptide antibodies were purified by affinity chromatography on peptide columns following assessment of their titers by ELISA as described previously (De Jongh et al., 1991). Preparation of the polyclonal antibody anti-CR2, raised against a fusion protein (CR2) corresponding to the final 549 amino acids of the cardiac α1 subunit, was described previously (Yoshida et al., 1992). Anti-CR2 IgG was purified by chromatography on protein A−Sepharose (PAS). All of these antibodies recognized their protein antigens specifically in ELISA analyses and recognized Ca^{2+} channels specifically in immunoprecipitation experiments and immunoblotting experiments. Prior incubation with the corresponding peptide antigen blocked recognition, and parallel experiments with nonimmune IgG gave no recognition.

Immunoprecipitation of [^{3}H]PN200−110-Labeled Ca^{2+} Channels. Rabbit cardiac membranes, prepared as outlined above, were incubated with 2 nM [^{3}H]PN200−110 at a final protein concentration of 2 mg/mL. Unbound label was removed by centrifugation at 120000*g* for 30 min at 4 °C and the labeled membranes were solubilized in 1% (w/v) digitonin, 10 mM Tris-HCl, pH 7.4, 1.5 mM $CaCl_2$, and 0.15 M NaCl with a 5:1 (w/w) detergent to protein ratio for 30 min at 4 °C. Insoluble material was removed by centrifugation at 120000*g* for 30 min at 4 °C. The total [^{3}H]PN200−110 bound to protein after solubilization was determined by diluting 100 μL of the solubilized material containing 200 μg of protein into 2.4 mL of buffer A. Bovine γ-globulin (500 μL of 10 mg/mL) was added, and the protein was precipitated by adding 1.5 mL of 30% (w/v) poly(ethylene glycol) (PEG, $M_r \sim 8000$) and incubating 15 min at 4 °C. Precipitated material was collected on GF/C filters which were then washed three times with 3 mL of cold 8.5% (w/v) PEG in 100 mM Tris-HCl, pH 7.4, before liquid scintillation counting.

[^{3}H]PN200−110-labeled, solubilized Ca^{2+} channels (80 or 400 μL) were diluted into 50 mM Tris-HCl, pH 7.4, 75 mM NaCl, 2.5 mM EDTA, 50 mM KF, and 20 mM sodium pyrophosphate (buffer D), containing 1 mg/mL BSA and 0.1% (w/v) digitonin, and incubated with various amounts of purified antibody in a final volume of 300 or 1500 μL, respectively, for 3 h at 4 °C with mixing by rotation. Immune complexes were precipitated by adsorption to PAS (5 mg/sample) for 20 min at 4 °C. Sepharose pellets were washed twice with buffer D containing 0.1% (w/v) digitonin and 1 mg/mL BSA prior to liquid scintillation counting. The amount of [^{3}H]PN200−110 precipitated was expressed as a percentage of the amount of bound compound added to each tube as determined by the PEG precipitation procedure outlined above.

Phosphorylation and Immunoprecipitation of Ca^{2+} Channels. Ca^{2+} channels were solubilized from cardiac membranes containing 2 pmol of PN200−110 receptor sites and purified on WGA−Sepharose as outlined above prior to incubation with antibody in 1% (v/v) Triton X-100 at 4 °C for 3 h. For immunoprecipitation of skeletal muscle Ca^{2+} channels, 0.5−10.0 pmol of purified channels were incubated with antibody in buffer D containing 1% (v/v) Triton X-100 and 1 mg/mL BSA at 4 °C for 3 h. Antigen−antibody complexes were subsequently adsorbed to 5 mg of protein A−Sepharose and washed four times with buffer D containing 1% (v/v) Triton X-100 and 1 mg/mL BSA and three times with 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, and 0.1% (v/v) Triton X-100 (buffer E). Phosphorylation was carried out by mixing the Sepharose pellets with 0.15 μM [γ-^{32}P]-ATP (3000 Ci/mmol) in buffer E. The reaction, initiated by the addition of 1 μg of the catalytic subunit of cA-PK, was carried out at 37 °C for 10 min and stopped by washing four times with buffer D containing 1% (v/v) Triton X-100 and 1 mg/mL BSA and once with buffer D.

For skeletal muscle Ca^{2+} channels, the pellets were incubated at 70 °C in SDS−PAGE sample buffer for 15 min prior to electrophoresis. For cardiac Ca^{2+} channels, the pellets were incubated at 70 °C in 50 mM Tris-HCl, pH 7.4, and 1% (w/v) SDS for 5 min and the supernatants were then diluted into 8 volumes of buffer D containing 1% (v/v) Triton X-100 and 1 mg/mL BSA. A second round of immunoprecipitation was carried out as outlined above, with the same antibody used for the first round of immunoprecipitation, and the washed pellets were incubated at 70 °C in SDS−PAGE sample buffer for 15 min prior to electrophoresis.

Polyacrylamide Gel Electrophoresis and Immunoblotting. SDS−PAGE was carried out according to Laemmli (1970) under reducing conditions on 6% (w/v) polyacrylamide gels for intact α1 subunits and 9% (w/v) acrylamide gels for the CR2 fusion protein. Electroblotting to nitrocellulose membranes was carried out in a Bio-Rad Mini Protean apparatus for 75 min at 100 V with 25 mM Tris-HCl, 192 mM glycine, and 20% (v/v) methanol, pH 8.3, as the transfer buffer. Unbound sites were blocked for 2 h at room temperature with 10% (w/v) skim milk powder in 10 mM Tris-HCl and 0.15 M NaCl, pH 7.4 (buffer F). Membranes were then incubated with antibodies in blocking buffer overnight at 4 °C, followed by six 5-min washes with buffer F containing 0.05% (v/v) Tween-20. Immunoreactive bands were visualized using the Amersham ECL immunoblotting detection system with horseradish peroxidase-linked protein A as the affinity reagent.

Two-Dimensional Phosphopeptide Mapping. Prior to two-dimensional mapping, cardiac Ca^{2+} channels were phosphorylated by cA-PK in the presence of [γ-^{32}P]ATP and immunoprecipitated as outlined above. The CR2 fusion protein was immunoprecipitated with anti-CR2 prior to phosphorylation. The reaction was stopped by the addition of SDS−PAGE sample buffer and incubation at 70 °C for 15 min. Following electrophoresis of phosphorylated samples as outlined above, wet gels were exposed to X-ray film to localize the ^{32}P-labeled cardiac Ca^{2+} channel α1 subunit or fusion proteins.

The method used for isolation and mapping of tryptic phosphopeptides was based on that outlined previously (Murphy & Catterall, 1992). Excised gel slices containing

phosphorylated α1 subunits or α1 fusion proteins were washed for 3 h and then 16 h in 10% (v/v) acetic acid/10% (v/v) 2-propanol followed by two 1.5-h washes in 50% (v/v) methanol (20 mL each wash). Slices were subsequently dried in a vacuum concentrator, rehydrated in 1.0 mL of 50 mM ammonium bicarbonate, pH 8.3, containing 50 μg of TPCK−trypsin, and incubated at 37 °C for 16 h. The supernatants were removed and the gel slices were washed by rotating with 1 mL of water for 2 h at 37 °C. Supernatants from each sample were combined and concentrated to 50 μL. The amount of ammonium bicarbonate was reduced by adding 500 μL of water and concentrating to 50 μL. This was repeated and the samples were evaporated to dryness. Tryptic phosphopeptides were resuspended in 1% (w/v) ammonium carbonate (pH 8.9) containing a trace of phenol red and subjected to electrophoresis for 45 min at 400 V, followed by ascending chromatography in 1-butanol/pyridine/acetic acid/water (15:10:3:12, v/v/v/v) on thin-layer cellulose plates. The plates were dried and subjected to autoradiography.

Phosphoamino Acid Analysis. Phosphorylated Ca^{2+} channel α1 subunit was trypsinized *in situ* as outlined above for two-dimensional phosphopeptide mapping. Acid hydrolysis and electrophoresis were carried out as outlined by Murphy and Catterall (1992).

Isolation of Phosphopeptides for Microsequence Analysis. CR2 fusion protein was phosphorylated by cA-PK in the presence of 0.2% (v/v) Triton X-100, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 0.9 mM [γ-^{32}P]ATP (1.32 Ci/mmol) for 1 h at 37 °C. The phosphorylated protein was isolated by SDS−PAGE and digested in excised gel slices with 2 μg of sequencing-grade modified porcine trypsin as outlined above. Tryptic peptides were recovered from the gel and purified by ferric chelate chromatography as outlined previously (De Jongh et al., 1993). Briefly, 700 μL of iminodiacetic acid−Sepharose was packed in a 1-cm diameter column and washed with 5 mL of each of the following solutions: water, 50 mM ferric chloride, water, and 0.1 M acetic acid, pH 3.0. The tryptic peptides were bound to the ferric-substituted Sepharose by rotation for 1 h at room temperature, and unbound material was collected. The Sepharose was washed with 0.1 M acetic acid, pH 3.0; 0.1 M acetic acid, pH 5.0; 0.1 M ammonium acetate, pH 7.4; 0.1 M ammonium acetate, pH 8.9; 0.1 M ammonium acetate, pH 10.0; and 0.1 M sodium phosphate, pH 8.0. Fractions (1.0 mL) were collected and the radioactivity in each was measured by Cerenkov counting. Fractions containing the highest radioactivity were combined and concentrated to 50 μL. The amount of salt was reduced by adding 500 μL of water and reconcentrating to 50 μL.

Trifluoroacetic acid (1% v/v) was added to the isolated phosphopeptides which were then separated on a Spheri-5 RP-18 C18 column (250 × 1.0 mm) with a flow rate of 80 μL/min. Gradient elution from 0.1% (v/v) trifluoroacetic acid to 48% (v/v) acetonitrile/0.085% (v/v) trifluoroacetic acid over 60 min was begun when the absorbance after injection of the sample had returned to baseline. Fractions were collected on the basis of absorbance at 206 nm into sterile Titer tubes (Bio-Rad). Fractions containing radioactivity were concentrated and rechromatographed on an Aquapore RP-300 C8 column (250 × 1.0 mm) under the same conditions. Amino acid sequences of fractions containing ^{32}P were determined with an Applied Biosystems Model 477A protein sequencer with an on-line model 120A phenylthiohydantoin (PTH) analyzer for amino acid identification. A sequence assignment of S′ indicates an increase of PTH−DTT−dehydroalanine, a derivative of phosphoserine, detected in the specified position compared to the previous step of Edman degradation.

Construction, Expression, and Phosphorylation of Recombinant Proteins. The CR2 protein, corresponding in sequence to the final 549 amino acid residues of the cardiac α1 subunit, was generated as outlined previously (Yoshida et al., 1992). The fusion protein CFH1−wt was generated from pCARD1 (Mikami et al., 1989) using the polymerase chain reaction (PCR). A 159-base-pair fragment containing amino acid residues 1895−1947 of the cardiac α1 subunit was amplified by PCR and cloned into the pGEX-3X expression vector (Pharmacia, Piscataway NJ) to obtain in-frame recombinant proteins containing glutathione S-transferase (GST). *Eco*RI and *Bam*H1 sites were included at the ends of the NH_2- and COOH-terminal oligonucleotides, respectively, to facilitate cloning. All constructs were verified by DNA sequencing and transformed into the protease-deficient strain BL26 of *Escherichia coli* (Novagen). Overnight cultures grown in 20 mL of YT medium supplemented with 100 μg/mL ampicillin (YT-amp) were used to inoculate 500 mL of YT-amp containing 0.4% (w/v) glucose and incubated at 37 °C for 3 h with shaking. Fusion protein synthesis was induced by the addition of 2 μM isopropyl β-D-thiogalactopyranoside, and the cells were cultured for an additional 2.5 h before being harvested by centrifugation at 5000*g* for 10 min . Cell pellets were resuspended in 10 mL of PBS containing 0.2 mM 4-(aminoethyl)benzenesulfonyl fluoride, 2 μM pepstatin A, 2 mg/ml Aprotinin, 2 μM leupeptin, 0.2 mM benzamidine, 0.5 mM EDTA, and 0.5 mM EGTA and the bacteria were lysed by mild sonication. The mixture was adjusted to 1% (v/v) Triton X-100, incubated 15 min on ice, and centrifuged at 10000*g* for 10 min. The supernatants were stored at −80 °C. The GST fusion proteins were purified from cell lysates by affinity chromatography on glutathione−Sepharose 4B according to the manufacturer's instructions (Pharmacia, Piscataway, NJ).

The fusion protein CFH1−S1928A is identical to CFH1−wt except that the serine residue corresponding to amino acid 1928 of the cardiac α1 subunit was mutated to an alanine residue using PCR mutagenesis (Ho et al., 1989). The fusion protein CaFSkα1−S1771/2A was generated as described in Rotman et al. (1995)

Purified fusion proteins were phosphorylated as described above in 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM dithiothreitol, and 0.1% (v/v) Triton X-100 with 0.15 μM [γ-^{32}P]ATP (3000 Ci/mmol). The reaction, initiated by the addition of 1 μg of cA-PK, was carried out at 20 °C for 1 min and stopped by heating in SDS−PAGE sample buffer at 65 °C for 5 min. ^{32}P-Labeled fusion proteins were analyzed by SDS−PAGE on 8.5% (w/v) porous-polyacrylamide gels (Doucet et al., 1990) or on 10−20% (w/v) Tricine gradient gels (Novex, San Diego CA) and located by autoradiography.

Phosphorylation of Ca^{2+} Channel α_1 Subunits in CCAR3217 Cells. CCAR3217 cells, derived from a CHO cell line expressing the α1 subunit of cardiac Ca^{2+} channels, were cultured as described by Yoshida et al. (1992). Cells were harvested by solubilization in 1% (v/v) Triton X-100 as

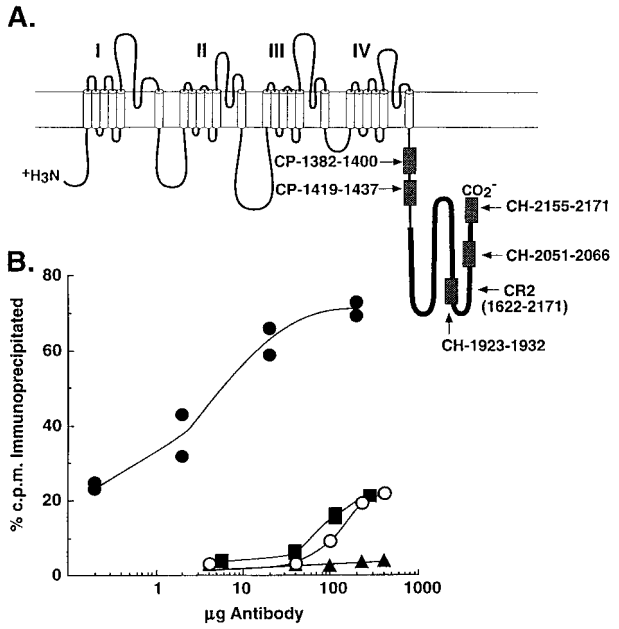


FIGURE 1: Location of antibodies and immunoprecipitation of the cardiac dihydropyridine receptor. (A) Peptides were synthesized corresponding to sequences (hatched boxes) in the COOH-terminal region of the cardiac α1 subunit. The peptides CP-(1382−1400) and CP-(1419−1437) were originally synthesized using sequences derived from the skeletal muscle Ca^{2+} channel α1 sequence. These sequences are completely conserved in the cardiac α1 sequence and correspond to residues 1507−1525 and 1543−1562, respectively. CR2 was generated as a fusion protein whose sequence corresponds to residues 1622−2171 of the cardiac α1 subunit. The position of CR2 is indicated by the bold line. (B) Rabbit cardiac membranes were labeled with [^{3}H]PN200−100, solubilized with digitonin, incubated with the indicated amounts of nonimmune IgG (filled triangles) or with antibodies directed against CH-(2155−2171) (filled squares), CH-(2051−2066) (open circles), or CR2 (filled circles) and precipitated with protein A−Sepharose. The radioactivity recovered in the precipitates is expressed as a percentage of the PEG-precipitable counts per minute.

described by Murphy et al. (1993), and Ca^{2+} channels were immunoprecipitated from cell extracts using anti-[CH-(2155−2171)]. To stimulate cAMP-dependent protein phosphorylation, cells were incubated in the absence or presence of the cAMP analogue DCl-cBIMPS (100 μM) for 10 min and harvested as described above in the presence of the phosphatase inhibitor microcystin (4 μM). Ca^{2+} channels were subsequently immunoprecipitated from cell extracts using anti-[CH-(2155−2171)] in the presence of 4 μM microcystin.

Immunoprecipitated Ca^{2+} channel proteins were then subjected to SDS−PAGE, transfered to nitrocellulose, and probed with anti-[CH-(1923−1932)-P] as described above. In some cases, blots were stripped by incubation in 2% (w/v) SDS and 62.5 mM Tris-HCl (pH 6.7) for 30 min at 50 °C, washed extensively in 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl, and reprobed with anti-[CH-(2155−2171)] as described above.

RESULTS

Immunoprecipitation of Cardiac Ca^{2+} Channels. In order to study the biochemical properties of the cardiac Ca^{2+} channel α1 subunit in sarcolemmal membranes isolated from rabbit heart, antibodies were generated against the epitopes illustrated in Figure 1A. These antibodies were used to determine the presence in the sarcolemmal membranes of

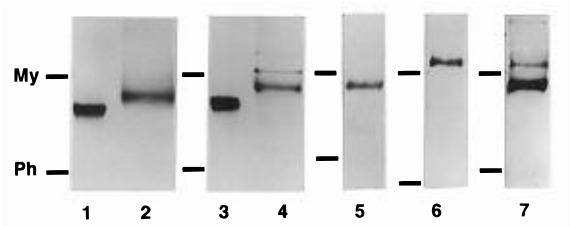


FIGURE 2: Immunoblot analysis of cardiac and skeletal muscle Ca^{2+} channel α1 subunits. Cardiac (lanes 2, 4, 6, and 7) or skeletal muscle (lanes 1, 3, and 5) Ca^{2+} channels were electrophoresed on 6% (w/v) acrylamide gels, transferred to nitrocellulose, immunoblotted with antibodies against CP-(1382−1400) (lanes 1 and 2), CP-(1419−1437) (lanes 3 and 4), CP-(1692−1707) (lane 5), CH-(2155−2171) (lane 6), or CR2 (lane 7), and visualized on X-ray film using chemiluminescent detection. The protein markers used were myosin (My, 200 kDa) and phosphorylase *b* (Ph, 97 kDa).

L-type Ca^{2+} channels having α1 subunits with specific epitopes by testing their ability to immunoprecipitate specifically bound [^{3}H]PN200−110, a high-affinity DHP calcium channel antagonist. Anti-CR2, directed against a fusion protein which corresponds to the final 549 amino acids of the cardiac α1 subunit, immunoprecipitated more than 70% of the [^{3}H]PN200−110-labeled Ca^{2+} channels solubilized from rabbit sarcolemmal membranes in the present study (Figure 1B). Affinity-purified antibodies directed against peptides corresponding to amino acid sequences 2051−2066 and 2155−2171 near the COOH terminus of the α1 subunit also specifically immunoprecipitated the [^{3}H]PN200−110-labeled Ca^{2+} channels solubilized from cardiac membranes (Figure 1B). However, in contrast to anti-CR2, these antibodies precipitated up to 20% of the [^{3}H]PN200−110-labeled L-type Ca^{2+} channels. These results suggested that the epitopes near the COOH terminus of the α1 subunit may be present only in a small portion of [^{3}H]PN200−110-labeled Ca^{2+} channels in this preparation.

Immunoblot Analysis of Cardiac Ca^{2+} Channel α1 Subunits. Two forms of the L-type Ca^{2+} channel α1 subunit are present in skeletal muscle, one of which is truncated at its COOH terminus between residues 1685 and 1699 (De Jongh et al., 1989, 1991). Antibodies directed against the peptides CP-(1382−1400) and CP-(1419−1437), which correspond to amino acid sequences before residue 1685 in the predicted skeletal muscle α1 subunit primary sequence, recognized the truncated form of the skeletal muscle α1 subunit, $\alpha1_{190}$ (Figure 2, lanes 1 and 3) Antibodies directed against a peptide corresponding to residues 1692−1707 of the skeletal muscle α1 subunit recognized the full-length form of the α1 subunit, $\alpha1_{212}$ (Figure 2, lane 5).

The amino acid sequences of CP-(1382−1400) and CP-(1419−1437) in skeletal muscle α1 are conserved in the cardiac α1 subunit, so the size of the α1 subunits solubilized from cardiac sarcolemma was examined by immunoblot analysis with the antibodies directed against these peptides. Anti-[CP-(1382−1400)] immunoreacted with a single protein of approximately 210 kDa (Figure 2, lane 2). Anti-[CP-(1419−1437)] also recognized this protein, as well as a protein of higher molecular mass which was present at lower concentration (Figure 2, lane 4). This larger protein's molecular weight was estimated to be approximately 240 kDa. Both the 210- and 240-kDa bands were also detected by immunoblotting with anti-CR2 (Figure 2, lane 7). These results clearly indicate that two size forms of the Ca^{2+} channel α1 subunit exist in sarcolemmal membranes.

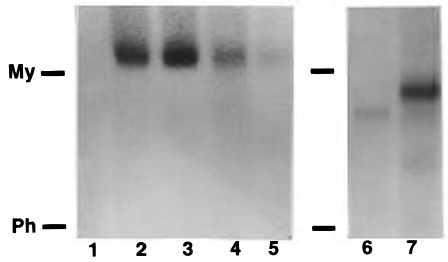


FIGURE 3: Immunoprecipitation of phosphorylated cardiac and skeletal muscle Ca^{2+} channel α1 subunits. Cardiac Ca^{2+} channels were immunoprecipitated with nonimmune IgG (lane 1) or with antibodies against CH-(2155−2171) (lane 2), CR2 (lane 3), CH-(2051−2066) (lane 4), or CP-(1382-1400) (lane 5). Precipitated α1 subunits were phosphorylated with [γ-^{32}P]ATP and cA-PK, released from the precipitate by heating in the presence of SDS, and subjected to a second round of immunoprecipitation using the same antibody as for the first immunoprecipitation. Skeletal muscle Ca^{2+} channels were immunoprecipitated with antibodies against CP-(1382−1400) (lane 6) or CP-(1692−1707) (lane 7) and phosphorylated with [γ-^{32}P]ATP and cA-PK. α1 subunits were released from the precipitates by heating in SDS−PAGE sample buffer and electrophoresed on 6% (w/v) acrylamide gels which were then exposed to X-ray film for visualization of incorporated ^{32}P. My, myosin; Ph, phosphorylase *b*.

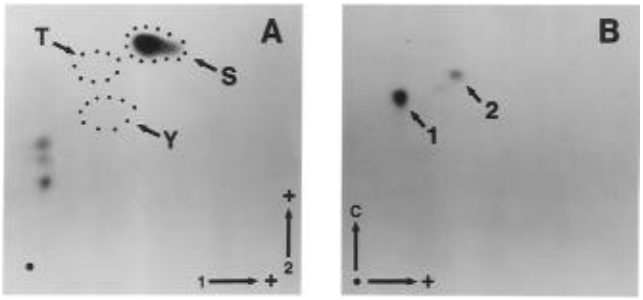


FIGURE 4: Two-dimensional phosphoamino acid and tryptic phosphopeptide analysis of cardiac Ca^{2+} channel α1 subunits phosphorylated by cA-PK. Immunoprecipitated cardiac Ca^{2+} channels were phosphorylated by cA-PK and analyzed by SDS−PAGE. ^{32}P-Labeled α1 subunits were located by autoradiography, excised from the wet gel, and processed for phosphoamino acid (A) or phosphopeptide (B) analysis as described in Experimental Procedures. (A) Acid-hydrolyzed samples were subjected to two-dimensional electrophoresis on thin-layer cellulose plates at pH 1.9 (first dimension), and at pH 3.5 (second dimension). A filled circle designates the origin, and dotted circles indicate the migration positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards, respectively. (B) Immunoprecipitated Ca^{2+} channel α1 subunits were phosphorylated by cA-PK and analyzed by SDS−PAGE. ^{32}P-Labeled α1 subunits were located by autoradiography, excised from the wet gel, and subjected to trypsin digestion as described in Experimental Procedures. Tryptic phosphopeptides were separated in two dimensions by high-voltage electrophoresis (pH 8.9), followed by thin layer chromatography. A filled circle designates the origin, and arrows designate the directions of electrophoresis (+) and chromatography (C). Visualized phosphopeptides were assigned a number (phosphopeptide 1 or 2) based upon their relative migration position.

Since the predicted mass of the cardiac α1 subunit deduced from its amino acid sequence is 242 771 Da, the approximately 240-kDa protein detected with both anti-CR2 and anti-[CP-(1419−1437)] likely represents the full-length cardiac α1 subunit, while the smaller protein of approximately 210 kDa detected with anti-[CP-(1382−1400)], anti-[CP-(1419−1437)], and anti-CR2 likely represents a truncated form of the α1 subunit. To examine this further, immunoblotting was carried out using an antibody raised against a peptide whose sequence corresponds to the final 17 amino acid residues of the predicted cardiac α1 sequence (anti-[CH-(2155−2171)]). This antibody immunoreacted with a single protein in the cardiac membrane preparation (Figure 2, lane 6) which migrated with the same electrophoretic mobility as the 242-kDa protein visualized with the CR2 antibody. These results indicate that only the 242-kDa protein contains the predicted COOH-terminal residues of the cardiac α1 subunit. Failure of anti-[CH-(2155−2171)] to immunoreact with the 210-kDa form of the α1 subunit indicates that this smaller form is truncated at its COOH terminus.

cAMP-Dependent Phosphorylation of Isolated Cardiac Ca^{2+} Channel α1 Subunits. Phosphorylation of cardiac α1 subunits solubilized from the membrane preparation was examined using a double immunoprecipitation protocol as described in Experimental Procedures, where phosphorylation by cA-PK in the presence of [γ-^{32}P]ATP was carried out following the first immunoprecipitation step. After a second round of immunoprecipitation and SDS−PAGE, ^{32}P-labeled proteins were visualized by autoradiography. No phosphoproteins were visualized when nonimmune IgG was used (Figure 3, lane 1). However, anti-[CH-(2155−2177)], anti-CR2, anti-[CH-(2051−2066)], and anti-[CP-(1382−1400)] all immunoprecipitated a single phosphoprotein (Figure 3, lanes 2−5) whose electrophoretic mobility was similar to that of the 242-kDa cardiac α1 subunit visualized in the immunoblots described in Figure 2. These results show that the 242-kDa, but not the 210-kDa, form of the cardiac α1 subunit is phosphorylated by cA-PK and suggest that the phosphate is incorporated into the COOH-terminal region of the full-length α1 subunit, which is absent in the truncated form.

Peptides Phosphorylated in the 242-kDa α1 Subunit. Phosphoamino acid analysis was carried out on isolated 242-kDa α1 subunits that had been phosphorylated by cA-PK to determine the identity of the phosphorylated residues. Only phosphoserine was detected (Figure 4A), indicating that phosphate was incorporated exclusively into serine residues.

Phosphorylation of the 242-kDa α1 subunit was examined further by two-dimensional tryptic phosphopeptide mapping as described in Experimental Procedures. Two phosphopeptides were observed (Figure 4B), with the intensity of phosphopeptide 1 greater than that of phosphopeptide 2. This result suggests that cA-PK incorporates phosphate into two major tryptic peptides derived from the cardiac α1 subunit.

Identification of the Sites of cAMP-Dependent Phosphorylation of the α1 Subunit by Protein Microsequencing. The low abundance of cardiac Ca^{2+} channels precluded direct microsequencing of the α1 subunit isolated from rabbit cardiac sarcolemmal membranes. We therefore employed the fusion protein CR2 (Yoshida et al., 1992), which could be prepared in quantities required for microsequencing. CR2 corresponds in sequence to the final 549 amino acids of the full-length cardiac α1 subunit predicted by cDNA cloning (Mikami et al., 1989; Slish et al., 1989). Purified CR2 was immunoprecipitated with anti-CR2, phosphorylated by cA-PK in the presence of [γ-^{32}P]ATP, isolated by SDS−PAGE, and subjected to two-dimensional tryptic phosphopeptide mapping as described in Experimental Procedures. Two major and two minor phosphopeptides were visualized (Figure 5). The two major phosphopeptides corresponded in migration position to phosphopeptides 1 and 2 in the peptide map derived from the intact cardiac α1 subunit

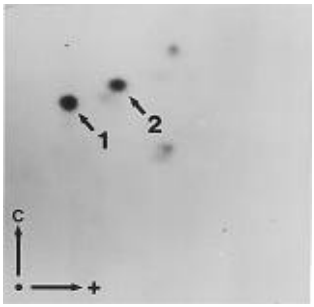


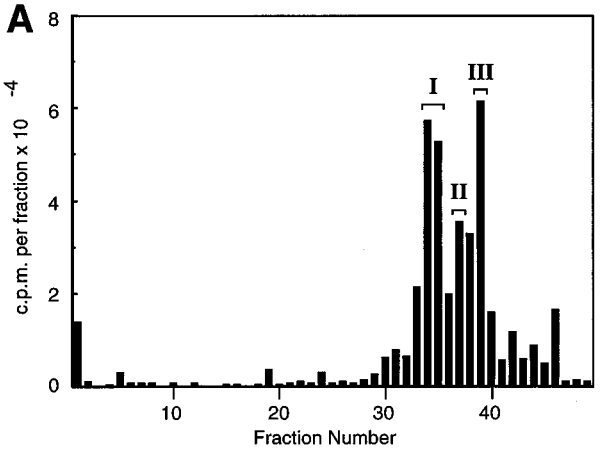
FIGURE 5: Tryptic phosphopeptide mapping of cA-PK-phosphorylated CR2 fusion protein. Immunoprecipitated CR2 fusion protein was phosphorylated by cA-PK and analyzed by SDS−PAGE. ^{32}P-Labeled CR2 was located by autoradiography, excised from the wet gel, and subjected to tryptic digestion as described in Experimental Procedures. Tryptic phosphopeptides were separated in two dimensions by high-voltage electrophoresis (pH 8.9) followed by thin layer chromatography. A filled circle designates the origin, and arrows designate the directions of electrophoresis (+) and chromatography (C).

(Figure 4B). These results are consistent with the conclusion that the same residues are phosphorylated by cA-PK in both CR2 and the full-length α1 subunit isolated from cardiac sarcolemma.

The identities of the tryptic phosphopeptides derived from CR2 were then determined using protein microsequencing. CR2 was phosphorylated by cA-PK , isolated by SDS−PAGE, and digested with trypsin, and the resulting phosphopeptides were purified by ferric chelate chromatography as described in Experimental Procedures. Subsequent reversed-phase HPLC on a C18 column resolved three radiolabeled phosphopeptide peaks (I, II, and III; Figure 6A). Each phosphopeptide peak was further purified by rechromatography on a C8 column, and the amino acid sequence of each was determined by Edman degradation. All three phosphopeptide peaks yielded the same NH_2-terminal amino acid sequence, which corresponds to residues 1926−1935 of the cardiac α1 subunit (Figure 6B). This sequence contains a single serine residue (serine 1928) located in a cA-PK phosphorylation consensus sequence. Phosphorylation at this position was suggested by recovery of PTH−DTT−dehydroalanine, the expected derivative of phosphoserine, at the position corresponding to serine 1928 in all three phosphopeptides.

Each purified phosphopeptide was also subjected to two-dimensional phosphopeptide mapping. HPLC peaks I, II, and III contained phosphopeptides that corresponded to phosphopeptides 2, 1, and 2, respectively, on the two-dimensional maps (data not shown). These results, together with the amino acid sequencing data, suggest that serine 1928 (Ser-1928) is the principal *in vitro* site of phosphorylation in the α1 subunit of the cardiac Ca^{2+} channel.

Identification of the Site of cAMP-Dependent Phosphorylation of the α1 Subunit by Fusion Protein Mutagenesis. The cA-PK phosphorylation site in the cardiac α1 subunit was also identified using a second fusion protein, CFH1−wt, corresponding to residues 1895 −1947 of the α1 subunit sequence linked to glutathione S-transferase. CFH1−wt was a good substrate for cA-PK (Figure 7A). The two-dimensional tryptic phosphopeptide map of ^{32}P-labeled CFH1−wt (Figure 7B) contained two major phosphopeptides corresponding in migration position to phosphopeptides 1 and 2 derived from intact cardiac α1 subunits and the CR2



B

HPLC peak	Amino acid sequence	Residue numbers	Phosphopeptide number
I	RAS'FHLEXLK	1926-1935	2
II	RAS'FHLEXLK	1926-1935	1
III	RAS'FHLEXLK	1926-1935	2

FIGURE 6: Identification of the site of cA-PK phosphorylation of the cardiac α1 subunit by protein microsequencing. (A) Tryptic phosphopeptides generated from the CR2 fusion protein were chromatographed on a C18 column as outlined in Experimental Procedures. Fractions were collected on the basis of their absorbance at 206 nm and the radioactivity in each was determined by Cerenkov counting. (B) The three peaks of radioactivity recovered from the C18 column (peaks I, II and III from panel A) were further purified by rechromatographing on a C4 column as outlined in Experimental Procedures. Each of the purified phosphopeptides obtained was subjected to NH_2-terminal sequence analysis. The sequences obtained, the corresponding amino acids in the Ca^{2+} channel α1 subunit sequence, and the migration position of each phosphopeptide in two-dimensional maps (refer to Figure 4B) are shown. S′ indicates PTH−DTT−dehydroalanine. No serine was seen at these cycles. X indicates no amino acid was observed. The cardiac α1 subunit contains a cysteine residue at this position, which was not detected during Edman degradation because cysteine is destroyed during acid hydrolysis unless it is first protected by chemical modification.

protein. These data are consistent with CFH1−wt being phosphorylated by cA-PK at the same site as CR2 and the α1 subunit.

CFH1−wt contains a single cA-PK consensus sequence surrounding the serine residue corresponding to amino acid 1928 of the cardiac α1 subunit. To confirm that this is the residue phosphorylated *in vitro*, an additional fusion protein (CFH1−S1928A) was prepared with the serine corresponding to residue 1928 mutated to an alanine residue. A Coomassie blue-stained SDS−polyacrylamide gel of equivalent amounts of CFH1−wt (Figure 8, lane 1) and CFH1−S1928A (Figure 8, lane 2) shows that both fusion proteins migrate as single protein bands. When this amount of CFH1−wt fusion was phosphorylated by cA-PK in the presence of [γ-^{32}P]ATP and analyzed by SDS−PAGE and autoradiography, a single ^{32}P-labeled phosphoprotein of the predicted molecular mass was detected (Figure 8, lane 3). When an identical amount of purified CFH1−S1928A was incubated in the presence of cA-PK and [γ-^{32}P]ATP and analyzed by SDS−PAGE, no ^{32}P-labeled phosphoproteins were detected (Figure 8, lane 4). These results show that the CFH1−wt fusion protein is a substrate for phosphorylation by cA-PK while CFH1−S1928A is not. These data

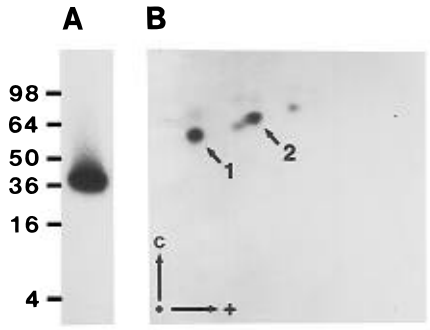


FIGURE 7: Identification and tryptic phosphopeptide mapping of cA-PK-phosphorylated CFH1 fusion protein. (A) Purified CHF1−wt fusion protein was phosphorylated in the presence of cA-PK and [γ-^{32}P]ATP and analyzed by SDS−PAGE as described in Experimental Procedures, and phosphoproteins were detected by autoradiography. Molecular weight markers are represented as $M_r \times 10^{-3}$. (B) ^{32}P-Labeled CHF1−wt was excised from the wet gel in panel A and subjected to tryptic digestion as described in Experimental Procedures. Tryptic phosphopeptides were separated in two dimensions by high-voltage electrophoresis (pH 8.9) followed by thin layer chromatography. A filled circle designates the origin, and arrows designate the directions of electrophoresis (+) and chromatography (C).

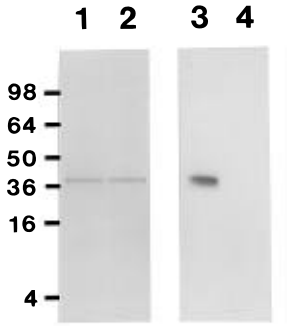


FIGURE 8: Phosphorylation of CFH1−wt and CFH1−S1928A fusion proteins by cA-PK. Cardiac fusion proteins CFH1−wt (lane 1) and CFH1−S1928A (lane 2) were purified as described in Experimental Procedures and 0.5 μg of each was analyzed by SDS−PAGE and stained with Coomassie Blue. Equivalent amounts of CFH1−wt (lane 3) and CFH1−S1928A (lane 4) fusion proteins were phosphorylated in the presence of cA-PK and [γ-^{32}P]ATP and analyzed by SDS−PAGE on a NOVEX 10−20% (w/v) Tricine gel and subjected to autoradiography as described in Experimental Procedures. Molecular weight markers are represented as $M_r \times 10^{-3}$.

are consistent with the identification of Ser-1928 as the principal residue phosphorylated by cA-PK *in vitro* in the α1 subunit of the cardiac Ca^{2+} channel.

Phosphospecific Antibodies to the Phosphorylation Site at Ser-1928. To directly examine the phosphorylation of Ser-1928 in the α1 subunit, a phosphospecific sequence-directed antibody was generated against the peptide [CH-(1923−1932)] encompassing the phosphorylation site at Ser-1928 (Figure 9A, sequence i) as described in Experimental Procedures. Figure 9B shows that anti-[CH-(1923−1932)-P] did not react with unphosphorylated CFH1−wt fusion protein (lane 1), whereas it reacted strongly with CFH1−wt fusion protein previously phosphorylated by cA-PK (lane 2). Anti-[CH-(1923−1932)-P] also did not react with mutant fusion protein CFH1−S1928A which had been previously incubated in the presence of cA-PK and ATP (data not shown). Together these results show that anti-[CH-(1923−1932)-P] specifically immunoreacts with the amino acid sequence surrounding Ser-1928 only when it is phosphorylated.

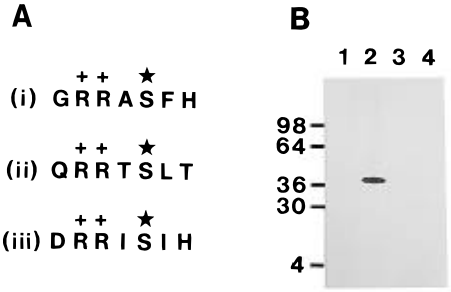


FIGURE 9: Immuoblotting of fusion proteins with the phospho-specific anti-peptide antibody anti-[CH-(1923−1932)-P]. (A) Amino acid sequence surrounding the cA-PK phosphorylation site in (i) the cardiac Ca^{2+} channel α1 subunit (Ser-1928), (ii) skeletal muscle Ca^{2+} channel α1 subunit (Ser-1757), and (iii) FP-13. Phosphorylated serine residues are indicated by the filled star. (B) Fusion proteins were phosphorylated in the presence of cA-PK and unlabeled ATP, analyzed by SDS−PAGE on a 10−20% (w/v) Tricine gel, and transferred to nitrocellulose as described in Experimental Procedures. Equal amounts of CFH1−wt (lane 1) and phosphorylated CFH1−wt (1 μg) (lane 2) were loaded on the gel. CaFSkα1−S1771/2A (lane 3) and FP-13 (lane 4) were titrated so that equivalent amounts of the phosphorylated form of each fusion protein were loaded on the gel relative to phosphorylated CFH1−wt. Blots were probed with anti-[CH-(1923−1932)-P] and visualized on X-ray film using chemiluminescent detection. Molecular weight markers are represented as $M_r \times 10^{-3}$.

To rule out the possibility that anti-[CH-(1923−1932)-P] reacts nonspecifically with phosphoserine in the context of any cA-PK consensus sequence, the antibody was tested against CaFSkα1−S1771/2A and FP-13, two unrelated fusion proteins which each contain a serine residue in a single cA-PK consensus sequence (Figure 9A, sequences ii and iii). Both sequences conform to the RRXS cA-PK consensus motif, as does Ser-1928 in the cardiac Ca^{2+} channel. CaFSkα1−S1771/2A (Rotman et al., 1995) and FP-13 (data not shown) are good substrates for cA-PK. Figure 9B shows that anti-[CH-(1923−1932)-P] did not immunoreact with phosphorylated CaFSkα1−S1771/2A (lane 3) or with phosphorylated FP-13 (lane 4). These results show that anti-[CH-(1923−1932)-P] specifically recognizes phosphorylated Ser-1928 in the context of the surrounding amino acid sequence. This phosphospecific sequence-directed antibody can therefore be used to determine the phosphorylation state of Ser-1928 in the cardiac Ca^{2+} channel α1 subunit.

Phosphorylation of Ser-1928 of the α1 Subunit in Mammalian Cells Expressing Cardiac Ca^{2+} Channel. CHO cells stably transfected with the rabbit cardiac α1 subunit (CCAR3217; Yoshida et al., 1992) were employed to examine cAMP-dependent phosphorylation of Ser-1928 in intact cells. The L-type Ca^{2+} channels expressed in these cells are effectively regulated by cA-PK (Yoshida et al., 1992; Sculptoreanu et al., 1993). To detect the cardiac Ca^{2+} channel α1 subunit, CCAR3217 cells were solubilized in 1% (v/v) Triton X-100, and the Ca^{2+} channels were specifically immunoprecipitated with anti-[CH-(2155−2177)]. Immunoprecipitated proteins were phosphorylated in the presence of cA-PK and [γ-^{32}P]ATP, analyzed by SDS−PAGE, and subjected to autoradiography as described in Experimental Procedures. Figure 10, lane 1, shows that anti-[CH-(2155−2177)] immunoprecipitated a single phosphorylated polypeptide of approximately 242 kDa. The migration position of this polypeptide is similar to that of the Ca^{2+} channel α1 subunit identified in extracts from rabbit sarcolemmal membranes (Figure 2). These results are in

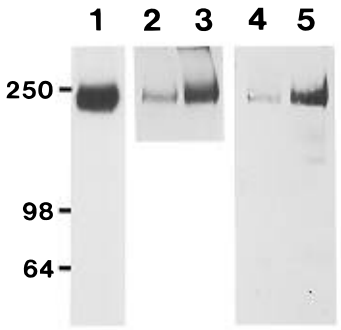


FIGURE 10: Phosphorylation of Ser-1928 in the α1 subunit of the cardiac Ca^{2+} channel in intact cells. (Lane 1) CCAR3217 cells were solubilized in Triton X-100 and Ca^{2+} channels were immunoprecipitated with anti-[CH-(2155−2177)]. Isolated channels were phosphorylated with cA-PK in the presence of [γ-^{32}P]ATP and analyzed by SDS−PAGE, and ^{32}P-labeled α1 subunits were visualized by autoradiography. (Lanes 2 and 3) CCAR3217 cells were solubilized in Triton X-100, and Ca^{2+} channels were immunoprecipitated with anti-[CH-(2155−2177)], incubated under phosphorylating conditions with unlabeled ATP in the absence (lane 2) or presence (lane 3) of cA-PK, analyzed by SDS−PAGE, and transferred to nitrocellulose. The blot was subsequently probed with anti-[CH-(1923−1932)-P] and visualized on X-ray film using chemiluminescent detection. Only the relevant molecular weight range was probed to conserve antibody. (Lanes 4 and 5) CCAR3217 cells were incubated in the absence (lane 4) or presence (lane 5) of DCl-cBIMPS as described in Experimental Procedures. Ca^{2+} channels isolated by immunoprecipitation with anti-[CH-(2155−2177)] were analyzed by SDS−PAGE and transferred to nitrocellulose. The blot was subsequently probed with anti-[CH-(1923−1932)-P] and visualized as described above. Molecular weight markers are represented as $M_r \times 10^{-3}$. Lanes 4 and 5 were quantitated by densitometric scanning using a Molecular Dynamics densitometer.

agreement with those of Yoshida et al. (1992); CCAR3217 cells express the full-length form of the cardiac Ca^{2+} channel α1 subunit and this is phosphorylated *in vitro* by cA-PK.

In a parallel series of experiments, CCAR3217 cells were solubilized in 1% (v/v) Triton X-100 and subjected to immunoprecipitation with anti-[CH-(2155−2177)]. Immunoprecipitated Ca^{2+} channels were incubated under phosphorylating conditions with unlabeled ATP in the presence or absence of cA-PK, resolved by SDS−PAGE, transferred to nitrocellulose, and analyzed by immunoblotting. Figure 10, lane 3, shows that anti-[CH-(1923−1932)-P] reacted with α1 subunits which had been incubated under phosphorylating conditions in the presence of cA-PK, consistent with our previous results which show that the α1 subunit isolated from cardiac sarcolemma is phosphorylated by cA-PK *in vitro* at Ser-1928. In contrast, anti-[CH-(1923−1932)-P] reacted very weakly with the α1 subunit that had been incubated in the absence of cA-PK (Figure 10, lane 2). This weak immunoreactivity may reflect a low basal level of phosphorylation of the Ca^{2+} channel in CCAR3217 cells.

To test if Ser-1928 was phosphorylated in intact cells in response to activation of endogenous cA-PK by elevation of intracellular levels of cAMP, CCAR3217 cells were incubated for 10 min in the presence or absence of the cell permeant cAMP analogue DCl-cBIMPS. The cells were solubilized in 1% (v/v) Triton X-100, lysates were subjected to immunoprecipitation with anti-[CH-(2155−2177)], and immunoprecipitated proteins were resolved by SDS−PAGE and electrotransferred to nitrocellulose. The blots were then probed with anti-[CH-(1923−1932)-P] to determine the phosphorylation state of Ser-1928. Figure 10 shows that anti-[CH-(1923−1932)-P] reacted strongly with Ca^{2+} channel α1 subunits in cells that were incubated in the presence of DCl-cBIMPS (lane 5) and weakly with α1 subunits in cells that were incubated in the absence of DCl-cBIMPS (lane 4). Densitometric scanning indicated that elevation of intracellular cAMP levels resulted in approximately a 6-fold increase in immunoreactivity. Since anti-[CH-(1923−1932)-P] specifically recognizes α1 subunits that have been phosphorylated at Ser-1928, these results demonstrate that Ser-1928 in the α1 subunit of cardiac L-type Ca^{2+} channels is phosphorylated in intact cells in response to an increase in intracellular levels of cAMP.

DISCUSSION

Molecular Properties of Two Size Forms of the α1 Subunits of Cardiac Ca^{2+} Channels. The results of the present study indicate that, like Ca^{2+} channels in skeletal muscle (De Jongh et al., 1989) and brain (Hell et al., 1993), the L-type Ca^{2+} channel α1 subunit isolated from heart muscle exists in two distinct size forms. Immunoblotting experiments have established that the longer form is similar in mass to the α1 subunit predicted from its cDNA sequence (242 771 Da; Mikami et al., 1989), and we therefore refer to this form of α1 as $\alpha1_{242}$. The smaller size form of the cardiac α1 subunit displays a similar electrophoretic mobility to skeletal muscle $\alpha1_{212}$, indicating that its apparent molecular mass is approximately 210 kDa. We therefore refer to this form of the cardiac α1 subunit as $\alpha1_{210}$. The failure of an anti-peptide antibody directed against the predicted final 17 amino acid residues of $\alpha1_{242}$ to immunoreact with $\alpha1_{210}$ indicates that this form of the α1 subunit is truncated at its COOH terminus. A molecular mass of 210 kDa is consistent with the COOH terminus of $\alpha1_{210}$ being near residue 1870 of the predicted α1 sequence. Similarly, the short form of the neuronal class C L-type Ca^{2+} channel α1 subunit isolated from brain has been shown to be truncated at its COOH terminus (Hell et al., 1993). Although the α1 subunit of class C brain channels shares 95% overall sequence identity with the cardiac channel, numerous amino acid substitutions are found from the end of transmembrane segment IVS6 to the COOH terminus (Snutch et al., 1991), and alternative splice products with different predicted phosphorylation sites in this region are expressed in brain and heart (Soldatov, 1994). Thus, the site and extent of truncation and the regulatory properties of the COOH-terminal region of the two proteins may be significantly different.

Both immunoblotting and immunoprecipitation experiments indicated that the major form of the α1 subunit present in Ca^{2+} channel preparations isolated from heart is truncated. It is likely that the 165−195-kDa α1 subunits isolated from heart in earlier studies (Ferry et al., 1987; Chang & Hosey, 1988; Schneider & Hofmann, 1988; Yoshida et al., 1990; Haase et al., 1991; Kuniyasu et al., 1992) represent the truncated form of α1. Our results do not distinguish whether the truncated form of the α1 subunit is present in intact cardiac muscle or is generated by proteolysis of full-length α1 subunit during channel isolation.

Site of Phosphorylation of the α1 Subunit of Cardiac L-type Ca^{2+} Channels. The present study shows that only $\alpha1_{242}$, and not $\alpha1_{210}$, isolated from rabbit heart is phosphorylated by cA-PK *in vitro*. These results are consistent with those of Yoshida et al. (1992), who showed that only a 250-

kDa form of the cardiac L-type Ca^{2+} channel α1 subunit heterologously expressed in CHO cells or isolated from cardiac muscle was a substrate for cA-PK. The fact that the COOH-truncated $\alpha1_{210}$ was not a substrate for cA-PK places the phosphorylation site/s in the COOH-terminal tail of the channel. Phosphorylation of $\alpha1_{242}$ at serine 1928 in the COOH-terminal tail was demonstrated by two approaches. Amino acid sequence analysis of three phosphopeptides derived from a fusion protein corresponding in sequence to the COOH-terminal 549 amino acids of $\alpha1_{242}$ yielded identical NH_2-terminal amino acid sequences corresponding to residues 1926−1935 of $\alpha1_{242}$. These results suggested that multiple phosphopeptides were generated by different trypsin cleavages around a single phosphorylated serine residue. Residue 1936 is arginine, so trypsin may have cleaved a portion of the CR2 polypeptides after this amino acid as well as after Lys-1935. Although each of the three phosphopeptides had an identical NH_2-terminal sequence, the low level of purified tryptic phosphopeptides available may have prevented detection of additional amino acids following lysine 1935. Since arginine is recovered from the sequencer in low yield and the final amino acid of a peptide is often poorly detected during N-terminal sequencing, it is possible that one or more of the phosphopeptides ended with arginine 1936. Phosphopeptide dimers connected by disulfide bridges involving the cysteine residue in these peptides may also produce multiple peptides which would yield the same NH_2-terminal sequence (Boyle et al., 1991). Whatever the source of heterogeneity in migration of the phosphopeptides that were sequenced, Ser-1928 was phosphorylated in each. Identification of this site of phosphorylation was verified by mutagenesis. The fusion protein CFH1−wt corresponding to amino acid residues 1895-1947 of $\alpha1_{242}$ was phosphorylated by cA-PK on the same tryptic phosphopeptides as $\alpha1_{242}$, indicating that the same residues were phosphorylated in each protein. Site-directed mutagenesis of Ser-1928 in CFH1−wt to alanine completely abolished phosphorylation of the fusion protein by cA-PK. This result confirms the identification of Ser-1928 as the *in vitro* site of phosphorylation of $\alpha1_{242}$.

Since Ser-1928 is in the COOH-terminal region of $\alpha1_{242}$, which is absent in $\alpha1_{210}$, our results provide a clear explanation for the lack of phosphorylation of the 210-kDa truncated form of the α1 subunit by cA-PK (Yoshida et al., 1992; and this paper). Since previous biochemical studies of purified cardiac Ca^{2+} channels employed preparations containing α1 subunits of 165− 195 kDa, the failure to demonstrate phosphorylation of α1 subunits in those studies is likely due to the lack of full-length α1 subunits in their preparations (Ferry et al., 1987; Chang & Hosey, 1988; Schneider & Hofmann, 1988; Yoshida et al., 1990; Haase et al., 1991; Kuniyasu et al., 1992).

Phosphorylation of Ser-1928 in Intact Cells. A critical step in evaluation of the mechanism of regulation by protein phosphorylation is demonstration that a specific residue is phosphorylated in intact cells under conditions which induce physiological regulation. CCAR3217 cells are a good model system for analysis of regulation of cardiac Ca^{2+} channels since cardiac α1 subunits expressed in them form L-type channels that are substantially regulated by cA-PK (Yoshida et al., 1992; Sculptoreanu et al., 1993). Elevation of intracellular levels of cAMP in CHO cells expressing the α1 subunit of the cardiac Ca^{2+} channel has been shown previously to result in the phosphorylation of a long form of the α1 subunit (Yoshida et al., 1992). Our results extend these observations and show that Ser-1928 in the COOH-terminal tail of the α1 subunit is selectively phosphorylated in these cells in response to increases in intracellular levels of cAMP. Phosphorylation of this site may contribute to modulation of the activity of cardiac Ca^{2+} channels by cA-PK and/or to regulation of the localization, expression, degradation, or protein−protein interactions of these channels.

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