Identification of the Sites Phosphorylated by Cyclic AMP-Dependent Protein Kinase on the β_2 Subunit of L-Type Voltage-Dependent Calcium Channels†

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ABSTRACT: Voltage-dependent L-type calcium (Ca) channels are heteromultimeric proteins that are regulated through phosphorylation by cAMP-dependent protein kinase (PKA). We demonstrated that the β_2 subunit was a substrate for PKA in intact cardiac myocytes through back-phosphorylation experiments. In addition, a heterologously expressed rat β_{2a} subunit was phosphorylated at two sites in vitro by purified PKA. This β_{2a} subunit contains two potential consensus sites for PKA-mediated phosphorylation at Thr_{164} and Ser_{591}. However, upon mutation of both of these residues to alanines, the β_{2a} subunit remained a good substrate for PKA. The actual sites of phosphorylation on the β_{2a} subunit were identified by phosphopeptide mapping and microsequencing. Phosphopeptide maps of a bacterially expressed β_{2a} subunit demonstrated that this subunit was phosphorylated similarly to the β_2 subunit isolated from heart tissue and that the phosphorylation sites were contained in the unique C-terminal region. Microsequencing identified three serine residues, each of which conformed to loose consensus sites for PKA-mediated phosphorylation. Mutation of these residues to alanines resulted in the loss of the PKA-mediated phosphorylation of the β_{2a} subunit. The results suggest that phosphorylation of the β_{2a} subunit by PKA occurs at three loose consensus sites for PKA in the C-terminus and not at either of the two strong consensus sites for PKA. The results also highlight the danger of assuming that consensus sites represent actual sites of phosphorylation. The actual sites of PKA-mediated phosphorylation are conserved in most β_2 subunit isoforms and thus represent potential sites for regulation of channel activity. The sites phosphorylated by PKA are not substrates for protein kinase C (PKC), as the mutated β_2 subunits lacking PKA sites remained good substrates for PKC.

Calcium influx through voltage-gated L-type calcium channels is responsible for initiating excitation−contraction coupling in the heart. The L-type calcium current in cardiac myocytes can be modulated by a variety of receptor-mediated processes including stimulation of the current through activation of the β-adrenergic receptor pathway (*1*−*4*). This pathway involves receptor-stimulated production of cAMP and activation of cAMP-dependent protein kinase (PKA).[1] While the cAMP-mediated stimulation of L-type Ca channels has been studied extensively by electrophysiological means (*4*, *5*), the underlying phosphorylation reactions have not been resolved completely.

L-type calcium channels are heteromultimeric proteins consisting minimally of pore-forming α_1 subunits and two accessory subunits, β and $\alpha_2\delta$, which play roles in modulating channel function (*6*−*11*). Several different isoforms of the α_1 and β subunits have been cloned, and many of these isoforms show cell-specific patterns of expression (*10*, *12*, *13*). The major subunits identified in cardiac tissue are the α_{1C} and β_2 isoforms that, together with the $\alpha_2\delta$ subunit, form the class C L-type Ca channel (*9*, *14*−*17*). Several splice variants of both the α_{1C} and β_2 subunits also exist and are believed to comprise class C L-type channels in other tissues such as smooth muscle and brain (*10*, *13*).

While direct phosphorylation of channel subunits seems a likely mechanism to explain the cAMP-dependent regulation of the channels, it has been difficult to study these reactions in intact cardiac myocytes because the channels are rare membrane proteins. Heterologous expression systems have been developed to overcome this problem, and both the expressed α_{1C} and the β_2 subunits of the calcium channel have been identified as excellent substrates for phosphorylation by PKA in vitro (*18*−*20*). Recent studies have provided evidence for a functional role of the phosphorylation of Ser1928 in the C-terminus of the α_{1C} subunit (*21*, *22*). However, two size forms of the α_{1C} subunit have been isolated from cardiac myocytes: the full-length form, which contains Ser1928, and a truncated form lacking a large portion of the C-terminus including Ser1928 (*15*, *17*, *23*, *24*). The truncated form of the α_{1C} subunit has consistently been the most abundant form isolated from cardiac preparations (*15*, *18*, *23*), and the C-terminal-truncated α_{1C} subunit is not

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[1] Abbreviations: C-subunit, catalytic subunit; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; WT, wild type; TPCK, tosylamido-2-phenylethyl chloromethyl ketone; Ab, antibody; IBMX, 3-isobutyl-1-methylxanthine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, (ethylenedinitrilo)-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; WGA, wheat germ agglutinin; PCR, polymerase chain reaction; BHK, baby hamster kidney; IVT, in vitro translated; TLC, thin-layer chromatography; CNBr, cyanogen bromide; HPLC, high-performance liquid chromatography; FPLC, fast-flow protein liquid chromatography; TFA, trifluoroacetic acid; IMAC, immobilized metal (ferric) affinity chromatography; CFTR, cystic fibrosis transmembrane regulator; Tn I, troponin I; PLB, phospholamban.

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a substrate for phosphorylation by PKA in vitro (*15*, *18*, *23*). These results suggest the possibility that Ser1928 may not be available to serve as a regulatory site in cardiac myocytes. On the other hand, results obtained from immunofluorescence studies have suggested that the C-terminus of the α_{1C} subunit is present in intact cardiac myocytes and colocalized with the body of the α_{1C} subunit and the β subunit (*17*). While further studies will be required to achieve a better understanding of the status and role of the C-terminus of the α_{1C} subunit in intact cells, it is conceivable that there may be multiple mechanisms whereby the L-type Ca channels are regulated by PKA. One scenario would predict that if the α_{1C} subunit in intact cardiac cells lacks the PKA target at Ser1928, then the regulation of channel function by PKA would occur through a mechanism other than phosphorylation of the α_{1C} subunit.

Since the β_2 subunit is a demonstrated PKA substrate both in vitro (*20*) and in intact cells (*22*), it is possible that PKA-mediated phosphorylation of the β_2 subunit in cardiac tissue may be functionally important. The rat β_{2a} subunit has been the β subunit most extensively studied in conjunction with the α_{1C} subunit [although the β_{2a} subunit appears to be expressed in the brain rather than the heart (*25*)]. This β_2 subunit contains two classical consensus sites for potential phosphorylation by PKA at Ser591 and Thr164 (*9*). The other β_2 isoforms have only one classical consensus site at residues homologous to Thr164 (*26*, *27*). This site corresponds to a homologous site in the skeletal muscle β_1 isoform, which has been shown to be phosphorylated by PKA in vitro (*28*). A β subunit has also been suggested to be phosphorylated in intact canine cardiac myocytes following β-adrenergic receptor stimulation (*29*, *30*). To determine if PKA-mediated phosphorylation of the β_2 subunit in cardiac myocytes may participate in the cAMP-dependent regulation of Ca currents, in the present work we tested for phosphorylation of the β_2 subunit in rabbit ventricular myocytes. In addition, we performed extensive analysis of the β_2 subunit to identify the sites of PKA-mediated phosphorylation.

MATERIALS AND METHODS

Materials. The catalytic (C) subunit of PKA was purified to homogeneity from bovine heart as previously described (*31*). Redivue [γ-^{32}P]ATP was purchased from Amersham Life Sciences. Tosylamido-2-phenylethyl chloromethyl ketone-(TPCK-)treated trypsin and clostripain were both obtained from Sigma. Lipofectamine was purchased from Qiagen. Prestained low range molecular weight electrophoresis standards were obtained from Gibco/BRL. Prestained high-range molecular weight electrophoresis standards, nitrocellulose, and Immobilon-CD membranes were purchased from Bio-Rad. Digitonin was from Gallard-Schlesinger or Calbiochem. [^{35}S]Methionine and [^{3}H]PN200−110 were obtained from Dupont NEN. Immunopure sulfo-NHS-LC-biotin, SuperSignal chemiluminescent substrate system, Ultralink immobilized protein G, Neutravidin, and biotinylated horseradish peroxidase (HRP) were purchased from Pierce. HRP-coupled secondary antibody was from Sigma, Pierce, or Jackson ImmunoResearch. All other reagents were from Sigma or other standard sources.

The polyclonal β_2 antibody was generated in rabbits against a fusion protein containing residues 462−600 of the rat β_{2a} carboxyl-terminus and specifically recognizes the β_2 isoform of this L-type Ca channel subunit (*32*). The polyclonal β_{gen} antibody was generated in a goat against a fusion protein containing residues 17−354 of the rat β_{2a} subunit and recognizes all identified L-type Ca channel β subunit isoforms (*33*).

Isolation of Rabbit Ventricular Myocytes. Adult rabbit ventricular myocytes were isolated by standard procedures (*34*) as previously described (*17*).

Back-Phosphorylation. For back-phosphorylation experiments, freshly isolated myocytes were kept at 37 °C for 1 h before drug treatment. Stimulated myocytes were treated with a combination of 20 μM forskolin (Calbiochem), 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma), 5 μM isoproterenol (Sigma), and 50 nM okadaic acid (OA, Calbiochem) for 5 min, while control myocytes were treated with vehicle alone. Membranes were prepared with a Tri-R dounce homogenizer (6−7 strokes at 7500 rpm) in lysis buffer containing 250 mM sucrose, 0.25 M KCl, 10 mM imidazole, pH 7.4, 5 mM $MgCl_2$, 10 mM EDTA, phosphatase inhibitors (100 mM NaF, 25 mM $NaKPO_4$, 2 mM EGTA, 10 mM sodium pyrophosphate, and 1 mM sodium vanadate) and protease inhibitors. Protease inhibitors consisted of the following: 184 μg/mL iodoacetamide, 17.4 μg/mL phenylmethanesulfonyl fluoride, 10 μg/mL soybean trypsin inhibitor, 1 μg/mL aprotinin, 1.4 μg/mL pepstatin A, and 10 μg/mL leupeptin. The homogenates were centrifuged at 5000*g* for 10 min. The pellets were resuspended in lysis buffer containing 0.6 M KCl to extract myosin and centrifuged again. Myosin extraction was repeated twice more, after which the pellets were washed with homogenization buffer containing 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, and phosphatase and protease inhibitors. Pellets were resuspended in a minimal volume of homogenization buffer and protein concentrations were determined prior to in vitro phosphorylation of the membranes. In vitro phosphorylation assays with PKA were performed on the crude membranes from the myocytes by methods previously described (*20*) with the exception that the specific activity of the [γ-^{32}P]-ATP was increased 50-fold by reducing the amount of unlabeled ATP in the reactions (from 50 μM to 1 μM).

After the back-phosphorylation, membranes were solubilized by addition of an equal volume of 2% SDS in homogenization buffer, and channel subunits were isolated by immunoprecipitation. Solubilized proteins were diluted 5-fold with dilution buffer (homogenization buffer containing 0.2% SDS, 0.8% digitonin, 0.25% cholate, and 0.2 M NaCl) and immunoprecipitated with the β_{gen} antibody precoupled to Ultralink protein G as described previously (*17*). Immunoprecipitation pellets were washed with 20 volumes of phosphoprotein wash buffer (1× homogenization buffer, 1% Triton X-100, and 0.2% SDS containing either 0.2 or 0.5 M NaCl) for a total of seven washes (starting and ending with 0.2 M NaCl wash). Precipitated proteins were eluted and analyzed as described below.

Purification of Cardiac L-Type Channels by Wheat Germ Agglutinnin−Sepharose Chromatography. Crude membranes from frozen rabbit hearts were prepared, and L-type Ca channels were prelabeled with [^{3}H]PN200−110 in the membranes, solubilized with 1% digitonin, and partially purified by wheat germ agglutinin (WGA)−Sepharose chromatography as previously described for channels from

avian heart (*15*). Fractions corresponding to the peak of dihydropyridine binding were concentrated either separately or after being pooled with Centricon-30 microconcentrators (Amicon) and analyzed by SDS−PAGE followed by Western blotting.

Expression of Cardiac Calcium Channel Proteins in Heterologous Systems. Mutants containing either truncations or point mutations were generated by PCR using the rat β_{2a} cDNA (*9*). The wild type (WT) and mutant cDNAs of the rat β_{2a} subunit were subcloned into the pRBG mammalian expression vector and used to express these proteins in BHK cells by Lipofectamine transfection per the manufacturer's recommended protocol (Qiagen). Cell membranes were prepared 48 h posttransfection. Cells were pelleted by centrifugation at 1000*g* for 10 min. The cell pellet was resuspended in ice-cold homogenization buffer. Cells were lysed with a Brinkman polytron homogenizer (power setting 5 or 6, three pulses of 10 s each) or a Tri-R dounce homogenizer (7−10 strokes, 7500 rpm). The homogenate was then centrifuged at 1000*g* for 5 min to remove nuclei and unbroken cells. The supernatant after this low-speed spin was centrifuged at 100000*g* for 30 min to separate the membrane/particulate fraction (pellet) from the soluble/cytosolic (supernatant) fraction. Finally, the pellet was resuspended in a minimal volume of homogenization buffer, protein concentration was determined, and the membranes were used in the in vitro phosphorylation experiments described below.

In vitro translated (IVT) WT and mutant β_{2a} subunits were expressed from cDNAs subcloned into the pCR3 vector and labeled with [^{35}S]methionine by use of the TNT-T7 quick coupled transcription and translation system (Promega). Bacterially expressed WT and mutant β_{2a} subunits were created with either the pQE30 6-His (Qiagen) or the pGEX-4T3 GST (Pharmacia) bacterial expression vector and purified as outlined by the manufacturer. Baculovirus-mediated expression of the β_{2a} subunit in Sf9 insect cells was performed as described earlier (*20*).

In Vitro Phosphorylation of Ca Channels. Membranes from BHK cells expressing the β_{2a} subunit or β_2 subunit partially purified from heart membranes by WGA−Sepharose were phosphorylated in vitro with PKA by methods previously described (*20*). The BHK membranes were then pelleted by centrifugation at 100000*g* for 30 min and resuspended in 500 μL of 2× phosphoprotein homogenization buffer (2× PHB) containing 40 mM Tris-HCl, pH 7.4, 100 mM NaF, 50 mM $NaKPO_4$, 10 mM EDTA, 10 mM EGTA, and 2× protease inhibitors. Membranes were solubilized in solubilization buffer (PHB, 0.1% SDS, 1% Triton X-100, and 0.5 M NaCl) for 30 min on ice. Insoluble material was removed by centrifugation at 5000*g* for 10 min. Solubilized membrane proteins or WGA-purified proteins were immunoprecipitated overnight with the β_{gen} antibody precoupled to immobilized protein G. The immunoprecipitation complex was pelleted by brief centrifugation and washed 5 times with at least 20 pellet volumes of cold solubilization buffer. The immunoprecipitated proteins were eluted by addition of 3 volumes of solubilization buffer and 1 volume of Laemmli buffer, heated at 95 °C for 5−10 min, and analyzed by SDS−PAGE, Western blotting, and autoradiography with a Storm phosphorimager.

In vitro phosphorylation of either the bacterially purified or the IVT β_{2a} subunit with either PKA or protein kinase C (PKC) was performed as described earlier (*20*) with the following exceptions. The protein concentration of the bacterially expressed β_{2a} subunit in the phosphorylation reaction was 0.1 mg of protein/ mL and the IVT β_{2a} subunit was phosphorylated in reactions containing 25 μL of the IVT reactions, 20 μL of 2.5× phosphorylation buffer (125 mM HEPES and 25 mM $MgSO_4$, pH 7.4) plus PKA C-subunit, ATP, and H_2O (to 50 μL total volume). The phosphorylation reactions on both the purified and the IVT β_{2a} subunits were stopped by addition of $^1/_5$ volume of Laemmli buffer, heated at 95 °C for 5−10 min, and analyzed by SDS−PAGE, Western blotting, and phosphorimaging.

SDS−Polyacrylamide Gel Electrophoresis and Immunoblotting. SDS−PAGE was performed by standard methods (*32*) under reducing conditions with various percentages of polyacrylamide gels as specified. Electroblotting to nitrocellulose or Immobilon-CD membranes was carried out according to previously described standard procedures (*32*). Membranes were incubated with primary antibody, either the β_2 antibody (*32*) at a concentration of 1:1000 or a biotinylated form of the β_{gen} antibody (*33*) at a concentration of 1:500, as specified for either 16 h at 4° C or for 5 h at 25 °C. Biotinylation of the β_{gen} antibody was performed with the sulfo-NHS-LC-biotin reagent (Pierce) according to the manufacturer's specifications. After the blot was rinsed with TBS (4 × 10 s/rinse), the appropriate secondary antibody was added and incubated for 1 h at 25 °C. The secondary antibody used for the β_2 blots was anti-rabbit Hrp-coupled antibody at a concentration of 1:4000, and the secondary system used for the biotinylated β_{gen} blots was precoupled Neutravidin (2.5 mg/mL) and biotinylated HRP (1.5 mg/mL). Membranes were washed with TBS [4 × (5−10 min/wash)] and visualized with SuperSignal chemiluminescent substrate for HRP-coupled antibodies.

One- or Two-Dimensional Phosphopeptide Mapping and Phosphoamino Acid Analysis. One-dimensional phosphopeptide mapping after cleavage with cyanogen bromide (CNBr) was performed as described (*35, 36*). Briefly, in vitro phosphorylated β_{2a} subunit expressed from recombinant baculovirus in Sf9 insect cells was separated by SDS−PAGE and transferred to Immobilon CD membranes. Phosphorylated β_2 subunit bands were cut from the transfer paper and subjected to cleavage by CNBr in 70% formic acid (500 μL) at room temperature for 5−8 h at the concentrations indicated. Following cleavage, the reactions were lyophilized and resuspended in SDS−PAGE sample buffer prior to electrophoresis on a 5−15% polyacrylamide gradient gel.

Two-dimensional phosphopeptide mapping was performed following cleavage with TPCK-trypsin (*37*) of in vitro phosphorylated, bacterially expressed WT and mutant β_2 subunits or the WGA−Sepharose-purified cardiac calcium channel β_2 subunit. Briefly, tryptic phosphopeptides of the β_2 subunit were separated by two-dimensional thin-layer chromatography (TLC) on cellulose F TLC plates (EM Separations). Separation was performed in the first dimension by electrophoresis in 1% $(NH_4)_2CO_3$, pH 8.9, and in the second dimension by ascending chromatography in *n*-butanol/pyridine/acetic acid/water (37:25:8:30). Phosphoamino acid analysis was performed on in vitro phosphorylated bacterially expressed WT β_2 subunits as described (*37*).

Purification and Microsequencing of Phosphopeptides. Bacterially expressed β_2 subunit was phosphorylated in vitro by PKA, separated by SDS−PAGE, and transferred to immobilon-CD membrane. The β_2 subunit bands were cut from the transfer paper and phosphopeptides were generated by cleavage with 500 μL of 50 μg/mL clostripain, 20 mM NH_4HCO_3, 2.5 mM DTT, and $CaCl_2$, pH 7.8, for 2−3 h at 25 °C. Cleavage reactions containing phosphopeptides were lyophilized and resuspended in buffer A [dH_2O with 0.1% (v/v) TFA]. Phosphopeptides were separated by reversed-phase HPLC on a C-18 column with buffer A (above) and buffer B (acetonitrile with 0.1% (v/v) TFA) in a linear gradient of 0−100% acetonitrile with 0.1% (v/v) TFA (buffer B). Fractions containing phosphopeptides were lyophilized and resuspended in buffer C (50 mM MES and 1 M NaCl, pH 5.5). Phosphopeptides were further purified by immobilized metal (ferric) affinity chromatography (IMAC) as described (*38*), lyophilized to near dryness, and desalted on a Sepharose G-10 gel-filtration column, and the purified phosphopeptides were microsequenced by Richard Cook at Baylor College of Medicine in Houston, TX.

RESULTS

Is the β_2 Subunit Isolated from Cardiac Myocytes Phosphorylated by PKA? To assess the ability of the cardiac β_2 subunit to serve as a substrate for PKA in intact cardiac cells, we asked if the β_2 subunit present in cardiac myocytes is phosphorylated in response to stimulation of the β-adrenergic receptor pathway. To do this, we stimulated intact rabbit ventricular myocytes with a cocktail of reagents to increase cAMP and subsequently performed back-phosphorylation experiments in isolated membranes as described under Materials and Methods. After immunoprecipitation of the β_2 subunit and SDS−PAGE, the β_2 subunit was detected by immunoblotting with the β_2-specific antibody, and phosphorylation of the β_2 subunit was determined by phosphorimaging. A single phosphorylated protein with an apparent molecular mass of approximately 68 kDa was detected in the immunoprecipitates and was recognized by the β_2-specific antibody (Figure 1A). The results of the back-phosphorylation experiments showed a marked decrease in radiolabeled phosphate incorporation from the in vitro phosphorylation reaction into the β_2 subunit when the protein was isolated from myocytes subjected to β-adrenergic receptor stimulation compared to the control condition (Figure 1A). This was consistent with the interpretation that elevations in cAMP levels in the intact myocytes resulted in increased phosphorylation of the β_2 subunit. Phosphate incorporation into the β_2 subunit isolated from stimulated myocytes was reduced by approximately 50% compared to the control conditions, suggesting a 2-fold increase in in vivo phosphorylation of the β_2 subunit following β-adrenergic receptor stimulation (Figure 1C). As a control for the back-phosphorylation technique, two known PKA substrates, phospholamban and troponin I (*39*−*41*), were also assessed for their abilities to be efficiently phosphorylated in response to β-adrenergic receptor stimulation under identical conditions (Figure 1B). Phospholamban and troponin I were also phosphorylated in vivo under these conditions, showing that the conditions used resulted in phosphorylation of expected substrates of PKA in these experiments (Figure 1B). These results demonstrated

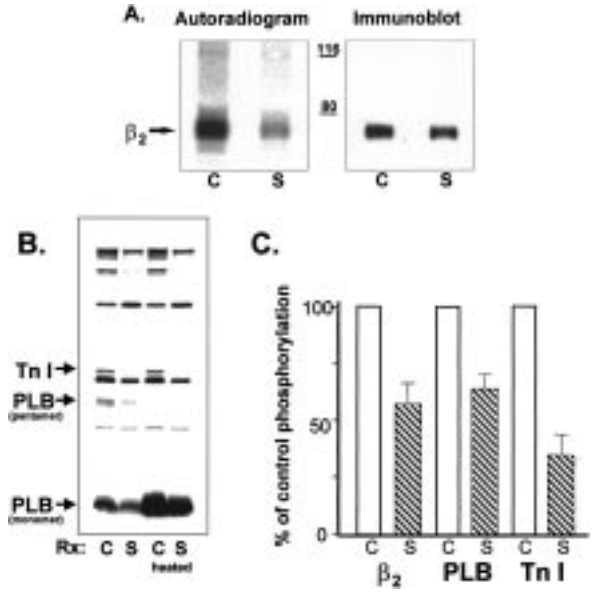


FIGURE 1: Native cardiac β_2 subunit isolated from rabbit cardiac myocytes is phosphorylated in vivo upon stimulation of PKA (A) Back-phosphorylation of the cardiac β_2 subunit. Isolated cardiac myocytes were subjected to back-phosphorylation as described under Materials and Methods. Stimulated myocytes (S) received drug treatment, while control cells (C) did not. The β_2 subunit was immunoprecipitated with the β_{gen} Ab and visualized by immunoblotting with the β_2-specific Ab (right panel) and by autoradiography (left panel). Protein markers shown are in kilodalton. **(B.)** Representative back- phosphorylation of the established PKA substrates, troponin I (Tn I) and phospholamban (PLB). Solubilized membrane proteins (10 mg) from control (C) and stimulated (S) myocytes were subjected to SDS−PAGE on a 15% acrylamide gel followed by staining with Coomassie blue (not shown) and analysis by phosphorimaging. In samples that were heated at 95 °C for 5 min, the characteristic dissociation of the pentameric form of PLB into monomers was observed. (C) Bar graph depicting relative phosphorylation of the β_2 subunit and the two control proteins, PLB and Tn I. Phosphorylation levels under control conditions for each protein were set at 100% and compared to phosphorylation levels under stimulated conditions. Levels of ^{32}P incorporation into the β_2 subunit were normalized for the differences in the amounts of protein immunoprecipitated (as determined by densitometric analysis of immunostaining). The results are the average of four independent experiments.

that the β_2 subunit was readily phosphorylated in vivo following β-adrenergic receptor stimulation in intact cardiac myocytes.

Mutation of the Classical PKA Consensus Sites in the β_2 Subunit Does Not Alter PKA-Mediated Phosphorylation. Since the cardiac calcium channel proteins are extremely rare membrane proteins, biochemical analysis of the native proteins is difficult. Therefore, to perform extensive biochemical analysis of the PKA-mediated phosphorylation reactions occurring on the cardiac β_2 subunit, heterologous expression systems were used to generate sufficient material for analysis and to allow for site-directed mutagenesis of the phosphorylation sites. The rat β_{2a} subunit contains two consensus sequences (*42*) for potential phosphorylation by PKA at Thr164 and Ser591 (*9*) (see Figure 2A). Point mutations of these residues to alanines were generated singly or together. The WT β_{2a} and the single (T164A or S591A) and double PKA consensus site point mutants were expressed in BHK cells. Membranes were prepared and phosphorylated in vitro with PKA. The phosphorylated β_{2a} subunits were immunoprecipitated with the β_{gen} antibody, separated by

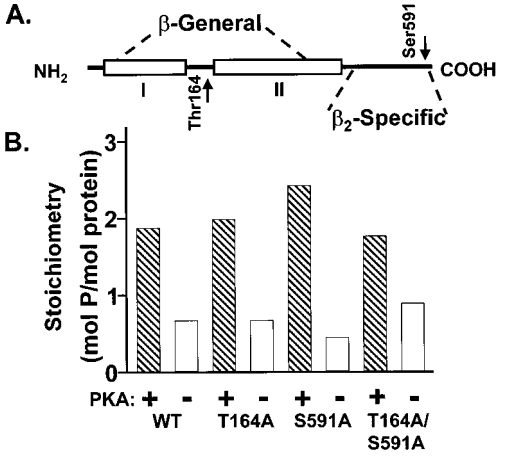


FIGURE 2: Classical consensus sites for PKA on the β_{2a} subunit are not the actual sites of phosphorylation. (A) Linear representation of the β_2 subunit delineating the epitopes used to generate the β_{gen} and the β_2-specific antisera, as described under Materials and Methods. Also shown is the location of the two classical consensus sites for PKA phosphorylation present in the rat β_{2a} subunit sequence (T164 and S591). (B) PKA phosphorylation of the rat β_{2a} subunit heterologously expressed in BHK cells. The WT β_{2a} subunit or mutant β_{2a} subunits lacking either one or both of the PKA classical consensus sites (T164A and S591A) were phosphorylated in vitro in the presence (+) or absence (−) of purified PKA as described under Materials and Methods. The bar graph shows stoichiometries of the PKA-mediated phosphorylation of the various β_{2a} subunits.

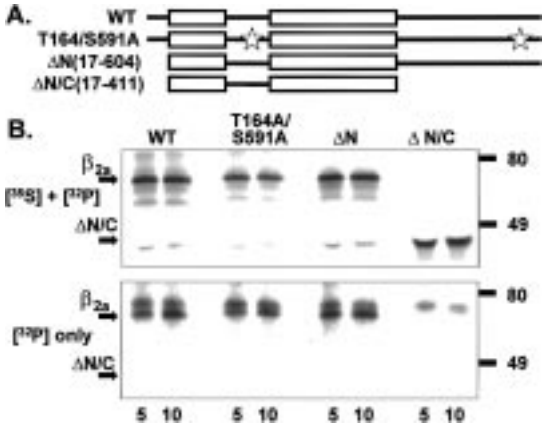


FIGURE 3: Sites of PKA phosphorylation are in the unique C-terminus of the β_{2a} subunit. (A) Schematic diagram of in vitro translated WT and mutant rat β_{2a} subunits. The PKA classical consensus sites (T164A/S591A) (stars) were mutated in one construct, and either the N-terminus (ΔN) or both the N- and C-termini (ΔN/C) were truncated in the other mutants. (B) WT and mutant β_{2a} proteins described in panel A were labeled with [^{35}S]methionine during in vitro translation, phosphorylated in vitro with PKA for either 5 or 10 min, separated by SDS−PAGE, transferred to nitrocellulose, and detected by autoradiography. The autoradiogram shows the doubly labeled β_{2a} proteins (^{35}S + ^{32}P; top panel) or only the ^{32}P signal following blockage of the ^{35}S signal with a thin sheet of plastic (bottom panel). Both the T164A/S591A mutant and the ΔN mutant were phosphorylated by PKA to a similar extent as the WT β_{2a} subunit, while the ΔN/C mutant was not a substrate for PKA in vitro. Protein markers shown are in kilodaltons.

SDS−PAGE, and visualized by immunoblotting with the β_2 antibody and by autoradiography. The stoichiometries of phosphorylation were determined by using a standard curve of an ^{35}S-labeled β_{2a} protein expressed by baculovirus in Sf9 cells as described earlier (*20*). The WT β_{2a} subunit was phosphorylated to a stoichiometry of about 2 mol of phosphate/mol of protein, and this was consistent with previous results obtained from in vitro phosphorylation of the baculovirus-expressed β_{2a} subunit (*20*). The results demonstrated that the β_{2a} mutants that lacked either one or both of the consensus sites for potential phosphorylation by PKA remained good substrates for phosphorylation by PKA (Figure 2B). These results suggested that the actual sites of phosphorylation by PKA on the β_{2a} subunit were not at the two consensus sites for potential phosphorylation by PKA. Alternatively, phosphorylation may have occurred at redundant sites in the mutant proteins.

The hypothesis that the classical consensus sites on the β_2 subunit for potential phosphorylation by PKA do not represent the actual phosphorylation sites was further tested. The WT β_{2a} protein and the double consensus site mutant were expressed in vitro by use of a reticulocyte lysate system. During expression the proteins were labeled with [^{35}S]-methionine in order to gauge their expression levels. The expressed proteins were phosphorylated in vitro with PKA and separated by SDS−PAGE, and both the ^{35}S and the ^{32}P signals were visualized by autoradiography. The ^{35}S signal was low energy and thus could be blocked by using a thin plastic sheet, while the ^{32}P signal was unaffected. Therefore, the ^{32}P signal, representing phosphate incorporation into the isolated proteins, was visualized by autoradiography when the ^{35}S signal was blocked (Figure 3B, bottom panel). The ^{35}S signal, representing the protein level, was determined by autoradiography without blocking the ^{35}S signal and subtraction of the ^{32}P signal determined above (Figure 3B, top panel). The distinct band for each protein could be visualized by its ^{35}S signal (top panel). An unknown, nonspecific protein present in the in vitro translation reaction was also phosphorylated under these conditions and was represented by the phosphoband separating at about 70−75 kDa, slightly above the phosphorylated, 68 kDa β_{2a} protein (bottom panel). When quantified, the levels of phosphate incorporation were similar for both the WT β_{2a} protein (lanes 1 and 2) and the double PKA consensus site mutant protein (lanes 3 and 4). Taken together with the above results, this suggested that the actual sites of PKA-mediated phosphorylation on the β_{2a} protein were at residues other than the two classical consensus sites for PKA.

Phosphopeptide Mapping and Phosphoamino Acid Analysis. To determine the actual sites of phosphorylation by PKA on the β_{2a} protein, further analysis was performed on the bacterially expressed β_{2a} protein. To demonstrate that the phosphorylation reactions occurring on the bacterially expressed β_{2a} protein and the β_2 protein isolated from cardiac tissue are similar, two-dimensional phosphopeptide mapping was performed. Both the bacterially expressed β_{2a} and WGA−Sepharose-purified, cardiac β_2 proteins were phosphorylated in vitro with PKA, separated by SDS−PAGE, transferred to Immobilon-CD membrane, and cleaved with trypsin. The resulting phosphopeptides were separated by TLC in two dimensions on cellulose plates and visualized by autoradiography. Both the bacterially expressed β_{2a} protein and the β_2 protein isolated from cardiac tissue generated similar phosphopeptide maps (Figure 4). While the phosphopeptide maps from both the native and the expressed β_2 proteins contained the same two major phosphopeptides (Figure 4), the phosphopeptide map of the bacterially expressed β_{2a} protein contained an additional, minor phos-

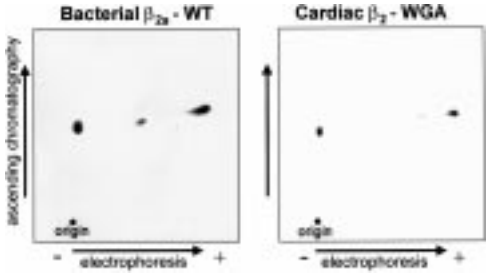


FIGURE 4: Two-dimensional phosphopeptide mapping of the β_2 subunit. Both the native cardiac β_2 subunit and the bacterially expressed rat β_{2a} subunit were phosphorylated with PKA, separated by SDS−PAGE, and transferred to Immobilon CD membranes. The ^{32}P-labeled β subunits were excised from the transfer membrane and subjected to trypsin digestion as described under Materials and Methods. Tryptic phosphopeptides were separated in two dimensions, high-voltage electrophoresis (pH = 8.9) in the first dimension and ascending thin-layer chromatography in the second dimension. Phosphopeptides generated from either the native cardiac β_2 subunit (right panel) or the bacterially expressed β_{2a} subunit (left panel) were visualized by autoradiography, and the identical migration of the two major phosphopeptides in both 2D maps demonstrated that the bacterially expressed β_{2a} subunit and the native cardiac β_2 subunit were phosphorylated at analogous sites. An additional, minor phosphopeptide present in the bacterially expressed β_{2a} subunit was later shown to represent the same site(s) as one of the two major phosphopeptides (see Figure 8B).

phopeptide (Figure 4). This minor phosphopeptide, however, was later shown to represent the same site(s) as one of the two major phosphopeptides (see Figure 8B). This result justified the use of the bacterially expressed β_{2a} protein for further analysis of the PKA-stimulated phosphorylation sites. Phosphoamino acid analysis was performed on the bacterially expressed β_{2a} protein, and this analysis indicated that serine was the sole phosphoamino acid (data not shown).

CNBr Phosphopeptide Mapping and β_{2a} Truncation Mutants Reveal the Importance of the Unique C-Terminal Region of β_{2a} for PKA-Mediated Phosphorylation. As an initial screen for the location of the actual residues phosphorylated by PKA, two truncation mutants of the β_{2a} protein were expressed in an in vitro translation system. The first of these mutants was truncated at the N-terminus, removing the first 17 amino acids (Figure 3A, ΔN) while the other mutant, containing only residues 17−411 (Figure 3A, ΔN/C), lacked both the N-terminus and a large portion of the C-terminus. While the ΔN mutant appeared to be phosphorylated by PKA to a similar level as the WT β_{2a} protein (Figure 3B, lanes 5 and 6), the ΔN/C mutant was not a substrate for PKA under these conditions as demonstrated by comparison of the high degree of ^{35}S signal to the absence of ^{32}P signal on the autoradiogram at the position of the ΔN/C protein (Figure 3B, lanes 7 and 8; compare upper panel to lower panel). As mentioned earlier, the nonspecific phosphoprotein separating at about 70−75 kDa was also present in the ΔN/C reactions. Thus, the C-terminal variable region of the β_2 protein appeared to contain the sites of PKA-mediated phosphorylation.

To gain additional information about the regions of the β_{2a} subunit that are phosphorylated by PKA, CNBr peptide mapping was used. CNBr selectively cleaves peptides at methionine residues, and generation of a predicted cleavage map of the β_{2a} subunit by CNBr reveals several potential peptides of various sizes that may be separated by one-dimensional gel electrophoresis (Figure 5A). Membranes

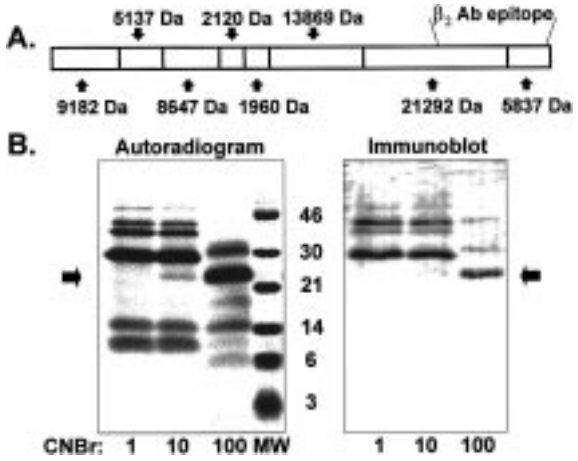


FIGURE 5: CNBr one-dimensional phosphopeptide mapping of the β_{2a} subunit phosphorylated with PKA. (A) Predicted cleavage map of the β_{2a} subunit using CNBr chemical cleavage showing the size of the predicted peptides generated by this method. The location of the epitope used to generate the β_2-specific antibody is indicated. (B) Membranes from Sf9 insect cells infected with a baculovirus expressing the WT β_{2a} subunit were phosphorylated with PKA, and phosphorylated β_{2a} subunits were separated by SDS−PAGE. ^{32}P-Labeled β subunits were excised from the gel and subjected to cleavage with increasing concentrations (1, 10, and 100 mg/mL) of CNBr as described under Materials and Methods. The phosphopeptides generated by this chemical cleavage method were analyzed on an SDS gel containing a gradient of 5−15% acrylamide and visualized by autoradiography (left panel) and immunoblotting with the β_2-specific antibody (right panel). A major phosphopeptide was generated with the highest concentration of CNBr (left panel, arrow), and this peptide was immunoreactive with the β_2-specific Ab (right panel, arrow) and, therefore, most likely represented the 21 292 Da peptide predicted in panel A. ^{35}S-Labeled protein markers shown are in kilodaltons.

from Sf9 insect cells infected with a recombinant baculovirus directing expression of the β_{2a} subunit (*20*) were phosphorylated in vitro with PKA. The β_{2a} protein was separated by SDS−PAGE, transferred to an Immobilon-CD membrane, and cleaved with CNBr. The resulting phosphopeptides were separated on an SDS gel containing a gradient of 5−15% acrylamide, transferred to nitrocellulose, and visualized by autoradiography (Figure 5B, first panel). The resulting phosphopeptide map showed that cleavage of the β_{2a} subunit with low concentrations of CNBr generated several phosphopeptides, most of which represented incompletely cleaved peptides. Use of the highest concentration of CNBr, however, generated one major phosphopeptide corresponding to a molecular mass of approximately 22−23 kDa, as well as several minor phosphopeptides (Figure 5B, left panel). Immunoblot analysis of the CNBr-generated peptides with the β_{2a} antibody showed immunoreactivity toward several phosphopeptides including the 22−23 kDa peptide, which contained the majority of the PKA-stimulated phosphorylation (Figure 5B, right panel). Since the β_{2a} antibody was generated against the C-terminal region of the β_{2a} subunit, the identity of the phosphopeptide separating at 22−23 kDa most likely corresponds to the C-terminal 21 292 Da peptide shown in the predicted cleavage map (Figure 5A). Thus, CNBr phosphopeptide mapping confirmed the results with the ΔN/C mutant and further identified the target of the PKA-mediated phosphorylation on the β_{2a} subunit to be in the C-terminus.

To further map the region of the β_{2a} subunit that is phosphorylated by PKA, several truncation mutants repre-

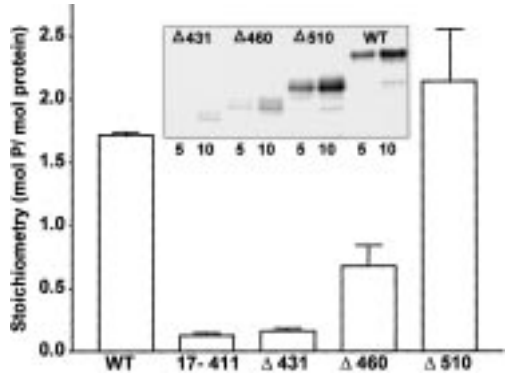
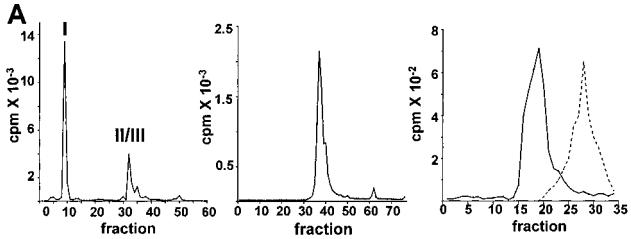


FIGURE 6: PKA-mediated phosphorylation of C-terminal trucation mutants of the β_{2a} subunit. Bacterially expressed C-terminal truncation mutants of the β_{2a} subunit were phosphorylated with PKA, separated by SDS−PAGE, and analyzed by autoradiography (inset panel) and immunoblotting with the biotinylated β_{gen} antibody (not shown). The bar graph represents the stoichiometries of PKA-mediated phosphorylation of the WT and mutant β_{2a} subunits after a 5 min phosphorylation reaction, and the results demonstrate that the Δ510 truncation mutant was phosphorylated to a similar level as the WT β_{2a} subunit, the Δ460 mutant was phosphorylated to approximately one-third the level of the WT β_{2a} subunit, and the Δ431 mutant had little detectable phosphorylation. The results are the average of four independent experiments.



B

HPLC peak	Amino Acid Sequence	Residue Numbers
I	RSSSATHQNHR	476-486
II/III	RSASQAEEEPCLEPVKKSQHR	456-476

FIGURE 7: Phosphopeptide purification and protein microsequencing. The bacterially expressed β_{2a} subunit was phosphorylated with PKA and cleaved with clostripain, and the resulting phosphopeptides were purified by HPLC and FPLC chromatography as described under Materials and Methods. (A) Elution profile of the phosphopeptides of the β_{2a} subunit (solid lines indicate ^{32}P radioactivity). The phosphopeptides were purified by reversed-phase HPLC on a C-18 column (left panel), further purified by IMAC (middle panel), which allowed for purification of phosphorylated peptides, and desalted (right panel) (dashed line = salt peak). Roman numerals (I, II, and III) show the ^{32}P peaks isolated from the C-18 column, which were each subsequently repurified on the IMAC column. The elution profiles for the IMAC and desalting columns were similar for all three samples isolated from the C-18 column. (B) Each of the three samples (I, II, and III) isolated from the purification steps in panel A was subjected to NH_2-terminal microsequencing analysis. The three samples revealed two distinct sequences corresponding to the indicated amino acids of the β_{2a} subunit sequence.

sented smaller C-terminal deletions were generated in bacteria. The WT and truncation mutants of the β_{2a} subunit expressed in bacteria were purified by nickel chromatography, phosphorylated with PKA, separated by SDS−PAGE, and detected by immunoblotting with a biotinylated form of the β_{gen} antibody and by autoradiography. The results showed that while both the WT β_{2a} protein and the Δ510 mutant of β_{2a} were phosphorylated to similar levels, the Δ460 mutant of β_{2a} showed a marked decrease in PKA-mediated phosphorylation (containing only about a one-third of the PKA phosphorylation level of the WT β_{2a} protein), and the Δ431 mutant of β_{2a} showed little or no phosphorylation (Figure 6). Thus, it appeared that the major sites of PKA-mediated phosphorylation of the β_{2a} subunit were contained within the C-terminus between amino acids 431 and 510.

Phosphopeptide Purification and Microsequencing. Purification and microsequencing of phosphopeptides were used to determine the actual sites phosphorylated by PKA on the β_{2a} subunit. The bacterially expressed β_{2a} subunit was phosphorylated with PKA, isolated by SDS−PAGE, transferred to Immobilon-CD membrane, and cleaved with the protease clostripain, which cleaves peptides C-terminal to arginine residues. The resulting phosphopeptides were initially purified by reversed-phase HPLC on a C-18 column (Figure 7A, left panel). Three major radioactive peaks resulted from this purification step, and each of these peaks was further purified by immobilized metal affinity chromatography (IMAC). Each of the three samples isolated from the HPLC purification behaved similarly and eluted from the IMAC column in a single radioactive peak (Figure 7A, middle panel). The final purification step involved desalting of each of the phosphopeptides on a Sephadex G-10 gel-filtration column, which also resulted in elution of a single radioactive peak (Figure 7A, right panel). Microsequencing analysis after Edman degradation revealed the amino acid sequences from two phosphopeptides, which corresponded to residues 456−476 and 476−486 in the rat β_{2a} subunit sequence (Figure 7B). These two sequences contained three serine residues each, serines 477−479 in one and serines 457, 459, and 473 in the other. Which of these residues was actually phosphorylated was not determined precisely from this procedure; therefore, site-directed mutagenesis was used to clarify this issue.

Loose Consensus Sites for PKA Represent the Actual Sites of PKA-Mediated Phosphorylation on β_{2a}. A review of the consensus sequences surrounding sites phosphorylated by PKA revealed that a third of the actual phosphorylation sites found in PKA substrates do not conform exactly to the classical consensus sequence, (R/K)(R/K)X(S/T) (*42*). Rather, these nonclassical sites are surrounded by a loose version of the classical consensus sequence which is missing either one of the two basic residues upstream of the phosphorylated residue (*42*). Examination of the sequences in the β_{2a} subunit that were identified by purification and protein sequencing revealed the existence of four loose consensus sites for PKA at serines 459, 473, 478, and 479. Therefore, these residues were mutated to alanines either singly or in groups on the basis of their proximity to one another, and the resulting mutant proteins were expressed in bacteria. The mutant proteins were purified by Ni chromatography, phosphorylated by PKA, isolated by SDS−PAGE, and detected by immunoblotting with the biotinylated β_{gen} antibody and by autoradiography. The phosphorylation levels of the bacterially expressed β_{2a} subunits were also compared with the baculovirus-expressed β_{2a} subunit, which has been shown previously to be phosphorylated under these conditions to a stoichiometry of approximately 2 mol of phosphate/mol of protein (*20*). A time course of phosphorylation by PKA demonstrated that both the bacterially expressed and the baculovirus-expressed WT β_{2a} subunits were phosphorylated to similar levels after a 5 min phosphorylation reaction

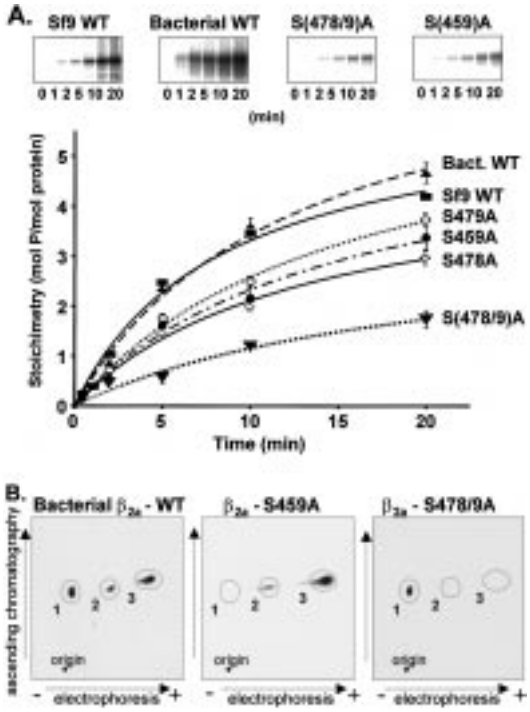


FIGURE 8: PKA phosphorylation of the β_{2a} subunit occurred at three loose consensus sites for PKA. (A) A time course of PKA-mediated phosphorylation of the bacterially expressed WT β_{2a} subunit and point mutants of the β_{2a} subunit, as well as the baculovirus-expressed WT β_{2a} subunit. The graph shows the stoichiometries of PKA-mediated phosphorylation of each of the WT and mutant proteins. The S459A mutant and the S478/9A double mutant, as well as the two single mutants S478A and S479A all showed significantly reduced levels of PKA phosphorylation (percent loss of PKA phosphorylation was 35% for S459A, 71% for S478/9A, 37% for S478A, and 28% for S479A at the 5 min time point). Point mutants of other residues in the C-terminus (S477A, S453A, S473A, S494A, T553A, and S570A) were phosphorylated by PKA to a similar extent as the WT β_{2a} subunit (not shown). The results are the average of five independent experiments. (B) Two-dimensional tryptic phosphopeptide mapping of the bacterially expressed WT β_{2a} subunit and the two point mutants, S459A and S478/9A, phosphorylated with PKA was performed as described under Materials and Methods. The S459A mutant showed a loss of one major phosphopeptide (middle panel), while the double mutant, S478/9, showed a loss of one major and one minor phosphopeptide (right panel) when compared to the WT β_{2a} subunit (left panel).

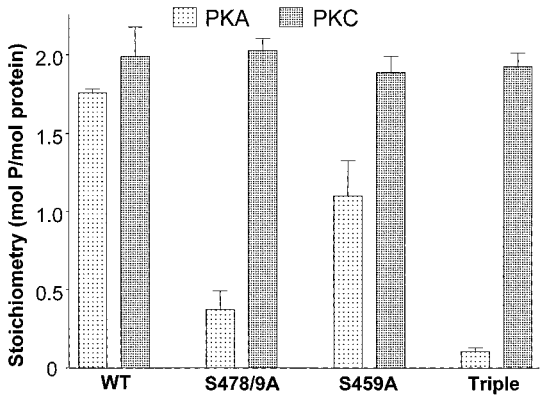


FIGURE 9: Point mutants of the β_{2a} subunit that exhibited reduced phosphorylation by PKA were phosphorylated to the same extent as the WT β_{2a} subunit by PKC. The bacterially expressed WT β_{2a} subunit and three point mutants of the β_{2a} subunit (S478/9A, S459A, and S459/478/9A [triple]) were phosphorylated in vitro with either PKA or PKC as described under Materials and Methods. The bar graph depicts the stoichiometry of phosphorylation by either kinase. These results demonstrate that the three point mutants of the β_{2a} subunit that were poor substrates for PKA were not grossly misfolded because they were phosphorylated by PKC to a similar extent as the WT β_{2a} subunit. The results are the average of four independent experiments.

(Figure 8A). However, the two β_{2a} mutants, S459A and S478/9A, were phosphorylated more slowly and to a lesser extent by PKA after a 5 min phosphorylation reaction (Figure 8A). While the single point mutant S477A was phosphorylated by PKA to a similar level as the WT β_{2a} subunit, the point mutants S478A and S479A both exhibited a reduction in phosphorylation to about a third the level of the WT β_{2a} subunit (Figure 8A). The double point mutant S478/9A (Figure 8A) was phosphorylated to about the same level as the triple point mutant S477−9A (not shown), both of which appeared to lose approximately two-thirds of the phosphorylation observed in the WT β_{2a} subunit.

Two-dimensional phosphopeptide mapping revealed the loss of a distinct subset of phosphopeptides for either of the two β_{2a} mutants, S459A and S478/9A, compared to the WT protein. Phosphorylation of the WT β_{2a} subunit by PKA resulted in one minor and two major phosphopeptides when cleaved with trypsin and separated by TLC (Figure 8B). The two-dimensional map of the S459A mutant showed loss of one of the major phosphopeptides, while the map of the S478/9A mutant demonstrated loss of the other two phosphopeptides (Figure 8B). The loss of two phosphopeptides in the double mutant could have been attributed to incomplete cleavage of one of the two phosphopeptides. Alternatively, this result could suggest that one of the two phosphopeptides observed was phosphorylated to a different stoichiometry than the other (i.e., one peptide had 1 mol of P/mol of peptide while the other had 2 mol of P/mol of peptide).

Taken together, these results demonstrated that residues S459, S478, and S479 represented the PKA phosphorylation sites on the β_{2a} protein. The two adjacent serine residues, S478 and S479, appeared to be phosphorylated synergistically because mutation of either residue by itself decreased phosphorylation but not to the same level as the double point mutant.

As a means to test that the bacterial proteins that were identified as mutants for PKA-mediated phosphorylation were folded similarly to the WT β_{2a} protein and that their lack of ability to serve as PKA substrates was not due to gross disruption of the protein caused by the mutations, the WT and mutant proteins were phosphorylated with PKC. We have previously demonstrated that the baculovirus-expressed WT β_{2a} protein was a good substrate for PKC and was phosphorylated to a stoichiometry of 1−2 mol of phosphate/mol of protein (*20*). We also have observed through CNBr phosphopeptide mapping that the majority of the PKC-mediated phosphorylation of the β_{2a} protein was incorporated into its unique C-terminal region (M. M. Hosey and B. L. Gerhardstein, unpublished results). The WT β_{2a} subunit expressed in bacteria was also a good substrate for PKC phosphorylation (Figure 9). While the three β_{2a} mutants, S478/9A, S459A, and S459/478/9A, were poor substrates for PKA, these mutants were readily phosphorylated by PKC and each to a similar extent as the WT β_{2a} subunit (Figure 9). These results suggested that the reduced ability of PKA to phosphorylate these mutant β_{2a} proteins did not simply result from gross misfolding of the mutant proteins since

the PKC-mediated phosphorylation of these proteins remained unaffected by the mutations. These results also demonstrated that the residues phosphorylated by PKC on the β_{2a} subunit were not the same as those phosphorylated by PKA.

DISCUSSION

We have demonstrated that the cardiac β_2 subunit is readily phosphorylated in intact cardiac myocytes following stimulation of the β-adrenergic receptor pathway. Another study has attempted to address the phosphorylation of a cardiac β subunit in intact dog heart; however, the identity of the phosphorylated protein was not verified by immunoblotting (*29*, *30*). In our back-phosphorylation experiments shown above, two antibodies were used, a general antibody to immunoprecipitate the β subunits from the heart and a β_2-specific antibody to confirm by immunoblotting the identity of the isolated phosphoprotein and to assess the amount of the β_2 subunit isolated in each immunoprecipitation. These results demonstrated that the β_2 subunit of the cardiac calcium channel is indeed phosphorylated in intact myocytes following β-adrenergic receptor stimulation.

Due to the exceedingly low amounts of channel proteins present in cardiac tissue and the inability to perform mutagenesis studies in the native system, heterologous expression systems were used to determine the sites of phosphorylation on the rat β_{2a} protein. Mutagenesis of the two classical consensus sites for potential phosphorylation by PKA on the β_{2a} subunit, Thr164 and Ser591, and subsequent phosphorylation of these mutants established the fact that these two sites were not the actual sites of phosphorylation by PKA on the β_{2a} protein.

Due to the lack of phosphorylation at the predicted PKA sites and the wealth of serines and threonines throughout the sequence of the β_{2a} subunit, the identification of the actual phosphorylation sites was complex and required a substantial effort employing classical biochemical methods as well as the creation of an array of potential phosphorylation site mutants. To identify the region of the β_{2a} subunit that was phosphorylated by PKA, we used phosphopeptide mapping, amino acid sequencing, and truncation mutagenesis. The results of CNBr phosphopeptide mapping, as well as phosphorylation of truncation mutants expressed in the in vitro translation system, both confirmed that the β_{2a} protein was phosphorylated by PKA in the C-terminus, downstream of the second conserved domain. Truncation mutagenesis of smaller regions of the C-terminus allowed us to map the sites phosphorylated by PKA to amino acids 431−510. Phosphopeptide purification and protein sequencing identified two peptide sequences that contained several serine residues. Mutagenesis of a single residue at Ser459 resulted in the loss of one site of phosphorylation by PKA, and mutagenesis of two residues at Ser478/479 resulted in the loss of approximately two sites of PKA-mediated phosphorylation. Mutagenesis of these three sites together caused a loss of the majority of the PKA-mediated phosphorylation on this protein. The location of these residues was in agreement with the truncation mutant data, which demonstrated that the two sites of phosphorylation by PKA were between amino acids 431−460 and 460−510. Sequence alignment of the different β_2 subunits resulting from splice variants and different species revealed that the serine residues at 459, 478, and 479 in the rat β_{2a} subunit sequence (*9*) are conserved in the other known β_2 subunits (Table 1) (*26*, *27*) with the exception of rabbit β_{2c}, in which the sequence for this region has been spliced out. These three serine residues are not conserved, however, in the β_1, β_3, or β_4 subunits (*10*, *12*, *13*), and this uniqueness may contribute to some of the distinct properties attributed to the β_2 subunits.

Table 1: Selected Sequence Alignment of the Various Isoforms and Species of the β_2 Subunit

Isoform	Selected Sequence Alignment
Rat β_{2a}	--RKST_{164}---RSAS_{459}--RS$SS_{478/9}$---KRRS_{591}-
Rabbit β_{2a}	--RKST_{165}---RSAS_{460}--RS$SS_{479/80}$--KKRN_{593}-
Rabbit β_{2b}	--RKST_{191}---RSAS_{486}--RS$SS_{505/6}$---KKRN_{619}-
Murine β_{2a}	--RKST_{171}---RSAS_{466}--RS$SS_{485/6}$---KRRN_{598}-

[a] The sequences of the β_2 subunit variants were aligned to show the similarities among the predicted classical consensus PKA sites and the actual PKA sites identified in the present work. While only the rat β_{2a} sequence contained the classical consensus site at S591, all of the sequences aligned contained identical residues surrounding the loose consensus sites identified as the actual PKA phosphorylation sites in Figures 7 and 8.

The classical consensus sequence for potential phosphorylation by PKA [(R/K)(R/K)X(S/T)] has been used to identify potential sites of phosphorylation by this kinase. However, in a review of both in vitro and in vivo substrates for PKA, it was shown that as many as a third of the actual sites phosphorylated by PKA on various substrates exhibited a loose consensus sequence for PKA and lacked one of the two upstream basic residues (*42*). Phosphorylation of the cystic fibrosis transmembrane regulator (CFTR) protein by PKA demonstrated this point. One of the major sites of PKA-mediated phosphorylation of the CFTR protein is at the loose consensus P-R-I-S (or X-R-X-S) (*43*). The three sites phosphorylated on the β_{2a} protein by PKA also represent loose consensus sequences. The sequence surrounding the PKA site at Ser459 is R-S-A-S (or R-X-X-S), and the sequence encompassing the PKA sites at Ser478/479 is R-S-S-S (or X-R-X-S and R-X-X-S at Ser478 and Ser479, respectively). The data obtained in this study, as well as in others where the sites of phosphorylation by PKA or other protein kinases do not conform to the commonly accepted consensus sequences, underline the potential misinterpretations that may arise when investigators mutate predicted consensus sites for PKA and test for functional defects without directly ascertaining whether the mutations led to actual defects in phosphorylation.

The β subunit plays many roles in controlling the function of L-type cardiac calcium channels. Coexpression of β subunits with α_1 subunits generates an increase in calcium currents over α_1 alone (*6*, *7*, *9*, *44*, *45*), and several studies have shown alterations of activation and inactivation kinetics (*8*, *46*) and a hyperpolarizing shift in the voltage dependence of activation of the current (*47*), depending upon the subunit isoform or type of expression system used. The importance of the β subunit has also been addressed by using antisense depletion of the various β isoforms from neuronal cells, which demonstrated a decreased peak current and a shifted voltage dependence of current activation (*47*). Considering

the importance of the β subunit to the function of L-type channels, it would not be surprising if this protein were also involved in the regulation of channel function by hormones and neurotransmitters. β-Adrenergic receptor stimulation causes a pronounced increase in the cardiac calcium current, as well as a hyperpolarizing shift in the peak current (*1*−*3*). These processes have been linked to the activation of PKA in cardiac cells; however, the actual substrates of PKA that allow for these events in intact cardiomyocytes have not been clarified. While both the full-length α_{1C} subunit and the β_{2a} subunit are substrates for PKA in heterologous systems (*20*, *22*), the actual substrates for PKA in the β-adrenergic activation of the cardiac calcium current remain unclear. The truncated α_{1C} protein isolated from cardiac muscle is not a substrate for PKA (*15*, *18*, *23*). Conceivably, regulation of the L-type channel may occur through multiple mechanisms (*11*), and the complexity of this system is only beginning to be unraveled. Here, we have demonstrated that one potential mechanism may involve phosphorylation of the β_2 subunit following stimulation of the β-adrenergic receptor pathway. While the identification of the phosphorylation sites was not a simple task, the new data that we have presented here will now allow for the design of future studies that will address the role of the β_2 subunit in the phosphorylation-dependent activation of the cardiac calcium channel by PKA in intact cells.

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