Differential Effects of Subunit Interactions on Protein Kinase A- and C-Mediated Phosphorylation of L-Type Calcium Channels[†]

Tipu S. Puri,[‡] Brian L. Gerhardstein,[‡] Xiao-Lan Zhao,[‡,||] Martha B. Ladner,[§] and M. Marlene Hosey*,[‡]

Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, Illinois, and Chiron Corporation, Emeryville, California

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ABSTRACT: We have expressed the pore-forming α_{1S} (skeletal muscle isoform) and α_{1C} (cardiac/brain isoform) subunits, as well as the accessory β_{2a} (cardiac/brain isoform) and α_2/δ subunits of the L-type, dihydropyridine-sensitive calcium (Ca) channels in *Spodoptera frugiperda* insect cells (Sf9 cells) by infection with recombinant baculoviruses in order to facilitate biochemical studies of these rare, heteromultimeric membrane proteins. Since the L-type channels are believed to be regulated by protein phosphorylation, this expression system allowed us to investigate which subunits could act as substrates for protein kinase A and C (PKA and PKC) and to determine the potential role of subunit interactions in phosphorylation of the channel proteins. Using purified protein kinases *in vitro*, the membrane-associated α_{1S}, α_{1C}, and β_{2a} subunits were demonstrated to be phosphorylated stoichiometrically by PKA. The extent of phosphorylation of these subunits by PKA was similar whether the subunits were expressed alone or in combination. In addition, the α_{1C} and β_{2a} subunits were phosphorylated stoichiometrically by PKC when expressed individually. In contrast, the α_{1S} subunit, when expressed alone, was a poor substrate for PKC, despite the fact that this subunit has been shown to be an excellent substrate for PKC in native skeletal muscle membranes. Interestingly, co-expression of α_{1S} with the β_{2a} subunit restored the ability of the α_{1S} subunit to serve as a substrate for PKC. These results strongly suggests that subunit interactions play an important and potentially differential role in channel regulation by PKC, whereas phosphorylation of the same subunit by PKA occurs independent of subunit interaction. Furthermore, our results provide biochemical evidence that, when co-expressed, the α_{1C}, α_{1S}, and β_{2a} subunits of L-type Ca^{2+} channels are excellent substrates for PKA and PKC and support the hypothesis that phosphorylation of each of these subunits may participate in channel regulation by these kinases.

L-type calcium (Ca) channels are voltage-dependent, heteromultimeric proteins that allow for Ca entry into a variety of cells (*1, 2*). Various isoforms of the pore-forming α_1-subunits have been identified and shown to complex with the "accessory" α_2/δ and β subunits that play critical roles in channel assembly and function. Many studies have shown that L-type channels can be modulated by neurotransmitters and hormones in a variety of tissues via receptor-mediated activation of signal transduction pathways involving protein kinases. Notably, in cardiac and neuronal cells extensive electrophysiological studies have supported the widely held hypothesis that phosphorylation of L-type channels by protein kinases is an important regulatory mechanism (*1, 2*). Indeed, the cardiac L-type channel, which is predicted to contain the α_{1C}, β_{2a}, and α_2/δ subunits, is a textbook example of an ion channel that is regulated by phosphorylation. However, detailed biochemical information regarding the protein phosphorylation reactions involved in the regulation of these L-type Ca channels is considerably limited because the channels are rare, complex membrane proteins that have been exceedingly difficult to study in native tissues. L-type channels are relatively more abundant in skeletal muscle, and the phosphorylation of the α_{1S} and β_1 subunits that are part of the skeletal muscle L-type channels has been characterized biochemically (*3*−*11*). However, there is still much to learn. For example, little is known about the role of accessory subunit interactions in kinase-mediated regulation. The structural and functional characteristics of subunits of L-type Ca channels in skeletal muscle differ significantly from those of their relatives in cardiac muscle and neurons. The presence of both similar and unique potential phosphorylation sites in the α_{1S} and α_{1C} subunits, as well as different accessory subunits that give rise to channel isoforms, suggests that both similar *and* unique reactions could be involved in the regulation of the isoforms of L-channels.

In addition to the technical difficulties that arise from the low abundance of L-type channels, another significant problem concerns the status of the carboxyl-terminal domains of the α_1-subunits. These domains of the channels are rich in potential phosphorylation sites, however they are often truncated in channels isolated from skeletal, cardiac, and neuronal tissues (*12*−*15*). The question still remains as to

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* To whom correspondence should be addressed at Department of Molecular Pharmacology and Biological Chemistry, S215, Northwestern University Medical School, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-3692. FAX: (312) 503-5349. E-mail: mhosey@nwu.edu.

[‡] Northwestern University Medical School.

[§] Chiron Corporation.

[||] Present address: Department of Pharmacology, University of California at San Diego, La Jolla, CA.

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whether the truncated forms of the proteins arise as a result of a post-translational processing event or as an artifact of cell lysis and protein isolation procedures. Phosphorylation studies of full-length α_1 subunits have been especially difficult because in most cases this form of the proteins, if detectable at all, has been observed to constitute only a very small percentage of the total native α_1 subunit in isolated membrane preparations (*13*−*16*). We have expressed the cDNAs for several components of cardiac and skeletal muscle L-channels in insect cells with recombinant baculoviruses in order to allow for detailed biochemical analyses of these rare membrane proteins. We have obtained new insights into which subunits may actually serve as substrates for protein kinases known to regulate L-channel function and novel information concerning the role of subunit interactions in protein phosphorylation.

MATERIALS AND METHODS

Viruses, Antibodies, and Other Reagents. Sf900II serum-free insect cell media (normal and methionine-free/cysteine-free) was purchased from Gibco/BRL. All other materials were from standard or previously described sources.

The cDNAs encoding the α_{1C}, β_{2a}, and α_2/δ subunits were generously provided by Drs. Lutz Birnbaumer, Arthur Brown, Ed Perez-Reyes, and Xiangyang Wei. To add either the KT3 T-antigen epitope (*17*) to the carboxy-terminus of the β_{2a} or α_2/δ subunits, or the GluGlu epitope (*18*) to the amino-terminus of the α_{1C} subunit, primer pairs were constructed which correspond to 18 nucleotides of the subunit coding sequence. One primer of each pair also contained the sequence encoding the appropriate epitope tag. DNA fragments containing the tag sequence were amplified by PCR before subcloning into the pAcC13 transfer vector. The α_{1C}, β_{2a}, and α_2/δ recombinant baculoviruses (α_{1C} BV, β_{2a} BV, and α_2/δ BV) directing expression of the tagged Ca channel subunits were generated using standard techniques (*19*). After co-transfection of Sf9 cells with wild-type viral DNA and the appropriate transfer vector, recombinant viruses were isolated by plaque purification (*20*). The α_{1S} BV was a generous gift of Dr. Linda Hall.

Epitope-tag directed and subunit-specific antibodies used in this study were designed against regions indicated in Figure 1. Generation and characterization of the subunit-specific antibodies Sk N, Sk I, Sk C, Card C, Card I, β_2, and β_{gen} have been previously described (*21*−*23*). The monoclonal GluGlu and KT3 antibodies (*17*, *18*) were generously provided by Dr. Gernot Walter. The polyclonal α170 antibody (*24*), which recognizes both the α_2 and δ peptides, was provided by Drs. Jacques Barhanin and Michel Lazdunski. The polyclonal alpha2 antibody was generated against a peptide comprising amino acid residues 102−115 located in the amino terminus of the α_2 peptide (*25*). Following peptide coupling to keyhole limpet hemocyanin (KLH) using standard protocols, the antibody was prepared in rabbit using the custom antisera production service of Bethyl Laboratories (Montgomery, TX).

Insect Cell Culture and Infection. Insect cells were grown either in suspension culture or monolayers in Sf900II serum-free media at 27 °C and infected using standard procedures (*19*, *26*).

Crude Membrane Isolation and Analysis of Expressed Subunit Proteins. Unless otherwise noted, infected Sf9 cells were harvested ∼72 h post-infection. Cells were pelleted by centrifugation at 1000*g* for 10 min and resuspended at 1 × 10^7 cells/mL in ice-cold homogenization buffer (Sf9HB) containing 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 5 mM EGTA, and protease inhibitors. Unless otherwise noted, "protease inhibitors" consist of the following: 184 μg of iodoacetamide (IAA)/mL, 17.4 μg of phenylmethylsulfonyl fluoride (PMSF)/mL, 10 μg of soybean trypsin inhibitor (SBTI)/mL, 1 μg of aprotinin/mL, 1.4 μg of pepstatin A/mL, and 20 μg of leupeptin/mL. Cells were mechanically lysed, and the homogenate was centrifuged at 1000*g* for 5 min to remove nuclei and unlysed cells. The supernatant was centrifuged at 100000*g* for 30 min to yield a crude membrane fraction (pellet) and a cytosolic (supernatant) fraction. The pellet was resuspended in Sf9HB and centrifuged a second time at 100000*g* for 30 min. The membrane fraction was finally resuspended in a minimal volume of Sf9HB. Crude membrane preparations were either used immediately for experiments or aliquoted and stored in liquid nitrogen until use.

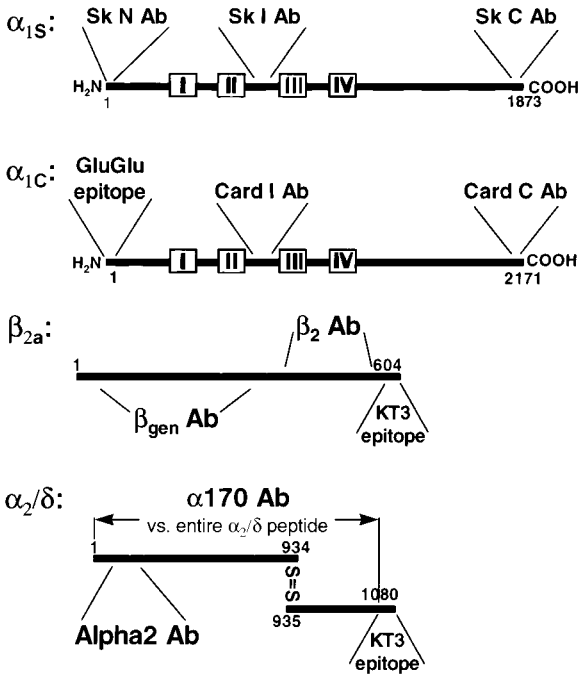

FIGURE 1: Key to subunit-specific and epitope-specific antibodies. Shown are the regions recognized by the α_{1S} (Sk N, Sk I, and Sk C), α_{1C} (Card I and Card C), β_{2a} (β_2 and β_{gen}), and α_2/δ (α170 and Alpha2) specific antibodies. Also shown are the positions of the GluGlu and KT3 epitopes on the α_{1C}, β_{2a}, and α_2/δ subunits.

SDS−PAGE was performed using standard methods (*27*). For non-reducing conditions, IAA was used in Laemmli loading buffer instead of 2-mercaptoethanol (βME) to give a final concentration of 8 mM IAA in samples. Western blot analysis, whole-cell [^{3}H]PN200-110 binding assays, and immunofluorescence staining of infected Sf9 cells were performed as previously described (*22*).

Biotinylation of Surface Proteins. Surface (plasma membrane) proteins of infected Sf9 cells were labeled with biotin, where indicated, immediately prior to isolation of crude membranes. Cells were gently pelleted (500*g*, 5 min) and resuspended at a concentration of 5 × 10^7 cells/mL in Sf900II media. Immunopure Sulfo-NHS-LC-Biotin (Pierce) was added to the cell suspension at a concentration of 0.56 mg/mL. Cells were rocked at room temperature for 30 min, and then crude membranes were isolated.

Metabolic Labeling of Expressed Proteins. Cells were infected as described above and after a 1−2 h incubation with virus, the innoculum was aspirated (monolayer cultures) or cells were gently pelleted (suspension cultures) at 500*g*, 5 min. Infected cells were then incubated with methionine-free/cysteine-free Sf900 media (19/20 of desired final media volume) for 1 h to deplete intracellular Met/Cys stores. Subsequently, 15−20 μCi/mL of Expre^{35}S^{35}S protein labeling mix (Dupont NEN) and 1/20 vol of normal Sf900II media were added. Cells were harvested after ∼72 h as described.

In Vitro Phosphorylation Studies. The catalytic subunit of protein kinase A was purified to homogeneity from bovine heart according to (*28*); protein kinase C was purified from avian brain as described previously (*29*). Crude Sf9 cell membrane preparations were phosphorylated *in vitro* with PKA and PKC using methods modified from those previously described for *in vitro* phosphorylation of channel proteins in rabbit skeletal muscle transverse-tubule preparations (*3, 4*). The differences from previous techniques were that membrane proteins were phosphorylated on ice for 30 min at 0.5 mg of protein/mL final concentration for PKA reactions and for 60 min at 0.2 mg of protein/mL final concentration for PKC reactions. Phosphorylation was tested at multiple temperatures, including between room temperature and 30 °C with no observed differences in the extent of phosphorylation. However higher temperatures did result in increased background phosphorylation in the "no kinase added" reaction probably as a result of activation of endogenous kinases present in the crude membrane preparation. Since crude membrane fractions were being used, incubations on ice for longer periods of time were used in order to minimize proteolysis and minimize background phosphorylation. Membranes were then pelleted by centrifugation at 100000*g* for 30 min and resuspended in 200 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, 2× protease inhibitors. Membranes were solubilized by addition of 200 μL of 2% SDS for 5 min followed by dilution to 0.2% SDS with phosphoprotein dilution buffer (PDB) containing 1× PHB plus 0.8% digitonin, 0.25% sodium cholate, 500 mM NaCl. Insoluble material was removed by centrifugation at 5000*g* for 10 min. Soluble proteins were immunoprecipitated overnight with the appropriate antibody (Card I for α_{1C}, Sk N for α_{1S}, and β_{gen} for β_{2a}) precoupled to immobilized protein G. The immunoprecipitation complex was pelleted by brief centrifugation and washed 5 times with at least 10 pellet volumes of cold PDB per wash. The immunoprecipitated proteins were eluted by addition of 3 vol of PDB and 1 vol of Laemmli buffer, heated at 95 °C for 5−10 min, and analyzed by SDS−PAGE, Western blotting, and phosphorimaging. With co-expressed subunit proteins, two sequential immunoprecipitations were used to isolate the subunit proteins. For example, in the case of co-expressed α_{1C} and β_{2a} subunits, the supernatant (non-precipitated proteins) after immunoprecipitation with the Card I antibody was subsequently incubated 4−12 h with the β_{gen} antibody precoupled to immobilized protein G. Immunoprecipitation complexes were washed, eluted, and analyzed as described.

For some experiments, membranes from biotin-labeled cells were used so that surface expressed α_1 subunit protein could be separated and analyzed. After immunoprecipitation and washing, precipitated proteins were eluted by incubation with an equal pellet volume of 4% SDS and 2% βME and heating at 95 °C for 5 min. The SDS and βME were diluted by addition of 9 vol of PDB to final concentrations of 0.2% and 0.1%, respectively. Eluted proteins were then precipitated by incubation with immobilized avidin overnight. The avidin precipitated proteins were washed, eluted, and analyzed as described above.

RESULTS

The objective of these studies was to establish an heterologous expression system that could facilitate biochemical studies of the phosphorylation of channel proteins. Before proceeding with phosphorylation studies, we determined if channel subunits expressed in insect cells exhibited their expected biochemical and functional properties. This was necessary as previous studies of a K^+ channel expressed with the baculovirus expression system yielded a largely insoluble, nonfunctional protein (*30, 31*).

Expression of the α_{1C} and α_{1S} Subunits. The α_{1C} subunit, believed to be the pore-forming channel subunits of L-type channels in heart, smooth muscle, and brain, was expressed with the GluGlu epitope at its amino-terminus (Figure 1) and recognized by the GluGlu antibody as a protein present in crude membrane preparations from α_{1C} BV infected cells (Figure 2a, lane 2) but not in preparations from non-infected cells (Figure 2a, lane 1). The molecular mass of the expressed α_{1C} subunit protein was calculated to be 240 kDa by Ferguson plot analysis (*32*) (data not shown). This result agrees well with the 242 771 Da molecular mass predicted for α_{1C} from its cDNA (*33*). Two additional antibodies, Card C and Card I, specific for carboxyl-terminal and internal regions of the α_{1C} protein respectively (see Figure 1), also recognized the same 240 kDa protein from α_{1C} BV infected cells (Figure 2a, lanes 3 and 4). Pre-immune antisera for Card I and Card C, as well as membrane proteins from non-infected or wild-type virus infected Sf9 cells, were non-reactive (data not shown). These results demonstrated that the α_{1C} BV directed expression of a full-length α_{1C} subunit protein. Further evidence that the epitope tag and α_{1C}-specific antibodies were reacting with the same expressed protein came from immunohistochemical studies. Dual-staining of α_{1c} BV infected Sf9 cells with both the GluGlu and Card C antibodies resulted in identical patterns of reactivity (Figure 2b). Peak expression of α_{1C} was determined to occur between 60 and 84 h post-infection (data not shown). In subsequent experiments, infected cells were harvested 72 h post-infection unless otherwise indicated. Both ionic and nonionic detergents, including digitonin (0.5−1.0%) and CHAPS (0.5−1.0%), proved capable of solubilizing a significant fraction of α_{1C} from the insect cell membranes (data not shown), in contrast to what was observed upon expression of a K^+ channel in Sf9 cells (*30, 31*).

We metabolically labeled α_{1C} BV infected Sf9 cells with [^{35}S]methionine and [^{35}S]cysteine in order to quantitate levels of expressed α_{1C} protein. After immunoprecipitation of the [^{35}S]α_{1C} with the Card I antibody varying amounts were analyzed by phosphorimaging and Western blotting with the Card C antibody (Figure 2c). By quantitation of the ^{35}S-label it was determined that Sf9 cells express approximately 2 pmol of α_{1C} per mg of crude membrane fraction, which is 10−50-fold higher than the amount expressed in heart and

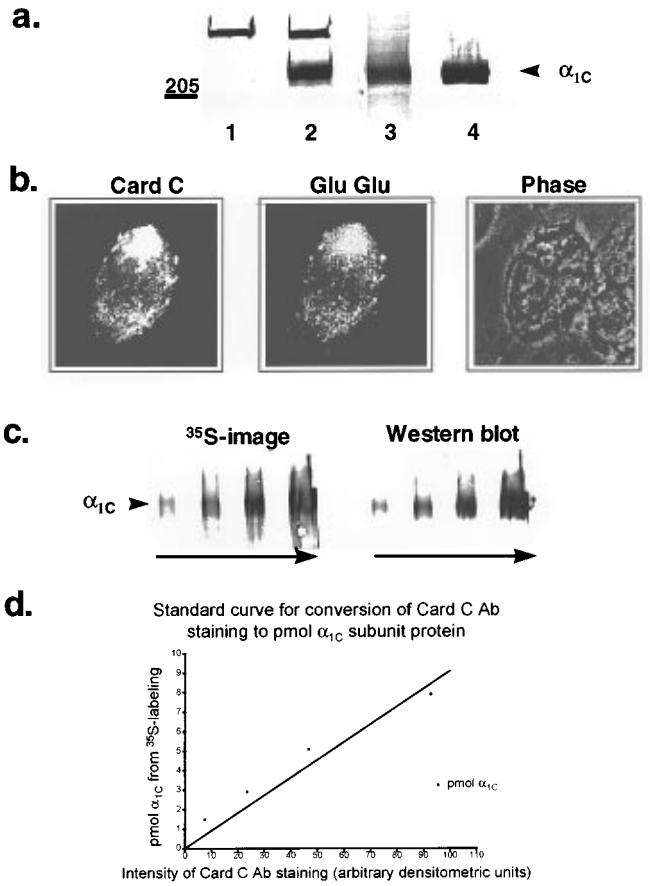

FIGURE 2: Expression of L-channel α_{1C} subunits in Sf9 cells. (a) Western blot analysis of crude membrane fraction from non-infected (lane 1) or α_{1C} BV infected (lanes 2−4) Sf9 cells. Shown is staining with the GluGlu (lanes 1 and 2), Card I (lane 3), and Card C (lane 4) antibodies. The GluGlu antibody also stained an ~270 kDa protein in both infected and non-infected cells that likely represents non-specific reactivity. (b) Immunohistochemical analysis of α_{1C} BV infected Sf9 cells stained with the Card C Ab (left) or the GluGlu Ab (center). Card C Ab staining was detected using fluorescein-tagged anti-rabbit secondary antibody and GluGlu Ab staining was detected using rhodamine-tagged anti-mouse secondary antibody. Light micrograph of stained cell is shown on the right. (c) After immunoprecipitation with the Card I Ab, increasing amounts (from left to right following arrows) of labeled α_{1C} subunit protein was analyzed by ^{35}S-imaging and immunostaining with the Card C Ab. (d) Standard curve generated from data in panel c relating picomoles of α_{1C} subunit to intensity of Card C staining.

brain. The quantitation of Card C immunostaining by densitometry allowed the generation of a standard curve relating Card C staining to pmol of α_{1C} subunit protein (Figure 2d), which was valuable for determining the stoichiometries of phosphorylation (see below).

In a similar fashion, α_{1S} BV infected cells expressed an ~185 kDa full-length form of the skeletal muscle α_{1S} subunit protein as assessed by Western blot analysis with the Sk N, Sk C, and Sk I antibodies (Figure 3, lanes 2, 4, and 6), which were generated versus the amino, carboxyl, and internal portions of α_{1S} (see Figure 1). Membranes from non-infected cells were unreactive (Figure 3, lanes 1 and 7). The α_{1S} cDNA predicts a protein of ~212 kDa (*34*). However, previous studies in native skeletal muscle have demonstrated that both full-length and truncated α_{1S} proteins migrate aberrantly on SDS gels (*16*). The expressed full-length α_{1S} migrated similarly to the ~190 kDa full-length native α_{1S} which was shown by Ferguson analysis to be 214 kDa (*16*). Indeed, the expressed α_{1S} migrated slower than the truncated

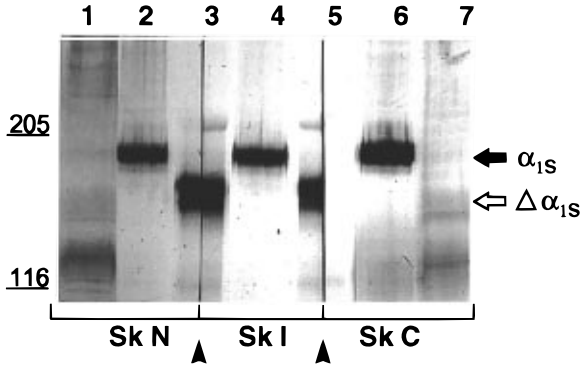

FIGURE 3: Expression of the L-channel α_{1S} subunit in Sf9 cells. Membrane fractions from non-infected cells (lanes 1 and 7), α_{1S} BV infected cells (lanes 2, 4, and 6), and rabbit skeletal muscle transverse tubules (lanes 3 and 5) are shown stained with the Sk N (lanes 1 and 2, and left-half of lane 3), Sk I (right-half of lane 3, lane 4, and left-half of lane 5), and Sk C (right-half of lane 5, and lanes 6 and 7) antibodies. Note that lanes 3 and 5 were cut down the middle (indicated by arrowheads) and each half-lane was stained with the indicated antibody. Arrows show the positions of the expressed (closed arrow) and native, truncated (open arrow) α_{1S} subunits.

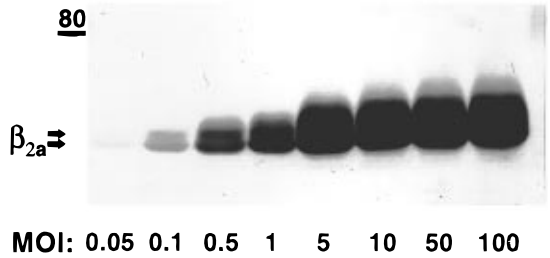

FIGURE 4: Expression of the L-channel β_{2a} subunit in Sf9 cells. Western blot of crude membrane proteins from cells infected with β_{2a} BV at increasing multiplicities of infection. Immunostaining is with anti-KT3 antibody.

form of α_{1S} in rabbit skeletal muscle transverse tubules (Figure 3, compare lanes 2, 4, and 6 with lanes 3 and 5). Note the truncated native α_{1S} reacted with the Sk N and Sk I antibodies but not with the Sk C antibody (Figure 3, lanes 3 and 5), confirming previous observations that it is truncated at the carboxyl-terminus (*13*, *16*, *21*). Thus the aberrant migration of the expressed α_{1S} at 185 kDa, which is below its predicted M_r, is consistent with that noted for the native α_{1S} protein (*16*).

Expression of the β_{2a} Subunit. The β_{2a} BV was designed to direct the expression of a KT3 epitope-tagged β_{2a} subunit protein (see Figure 1). On Western blots, monoclonal anti-KT3 antibodies recognized an ~70 kDa protein in crude membrane fractions from β_{2a} BV infected Sf9 cells (Figure 4) but not from non-infected cells (data not shown). The molecular mass of the expressed β_{2a} protein agreed well with that predicted by the β_{2a} cDNA, 68 191 Da (*35*). However, all of the expressed protein was found to be associated with the particulate fraction; at lower multiplicities of infection (MOIs), increased resolution of immunostaining revealed multiple isoforms of the expressed β_{2a} subunit protein (Figure 4) as has been observed previously in mammalian expression systems (*22*). At higher MOIs, the β_{2a} subunit was expressed at very high levels and was readily visualized as a major band after Coomassie Blue staining of membrane proteins (data not shown). Quantitation of β_{2a} subunit expression by ^{35}S-labeling (shown in Figure 7) revealed that Sf9 cells

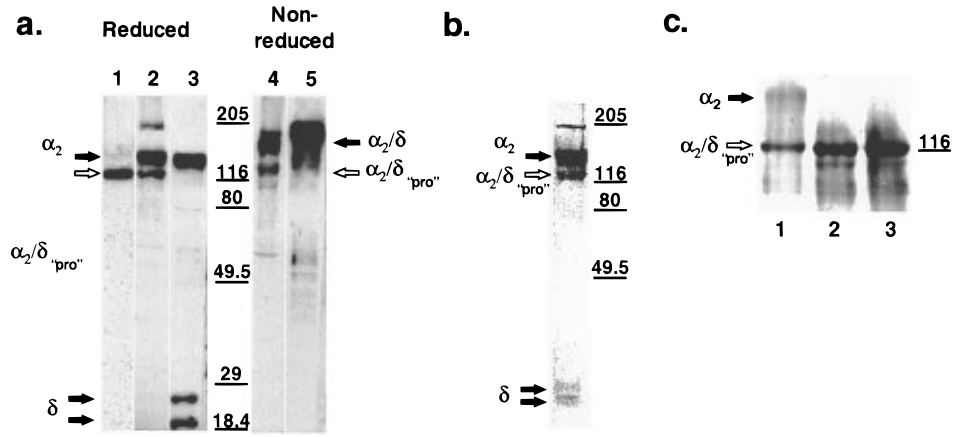

FIGURE 5: Expression of L-channel α_2/δ subunit in Sf9 cells. (a) Western blot analysis of expressed and native α_2/δ subunit after reducing (lanes 1−3) and non-reducing (lanes 4 and 5) SDS−PAGE. Lane 1, expressed α_2/δ subunit with anti-KT3 Ab staining. Lanes 2 and 4, expressed α_2/δ subunit with α170 Ab staining. Lanes 3 and 5, native α_2/δ subunit from rabbit skeletal muscle t-tubule with α170 Ab staining. In both panels the proposed "pro-form" of the α_2/δ subunit (open arrow) and the "mature", processed peptides (closed arrows) are indicated. (b) Western blot showing staining of expressed δ subunits. Expressed α_2/δ subunit was subjected to reducing SDS−PAGE followed by Western blot analysis using the α170 Ab. Stained protein bands are labeled as described for panel a. (c) Western blot showing effects of tunicamycin treatment on expression of α_2/δ subunit in Sf9 cells. Cells were infected with α_2/δ BV and either left untreated (lane 1) or incubated with 10 mg of tunicamycin/mL at 22 h (lane 2) or 46 h (lane 3) post-infection. Crude membrane proteins were isolated at 70 h post-infection and subjected to Western blot analysis with the alpha2 Ab. Stained protein bands were labeled as described for panel a.

express up to 200 pmol of β_{2a} protein/mg of crude membrane proteins.

Expression of the α_2/δ Subunit. The native α_2/δ subunit is the product of a single gene which predicts a protein of ∼120 kDa (*25*, *36*, *37*). Evidence suggests that the product of the α_2/δ gene undergoes extensive post-translational processing resulting in a heavily glycosylated α_2 peptide disulfide bonded to a membrane-anchoring δ peptide (*36*−*38*). The α_2 and δ peptides are separated under the reducing conditions normally used in SDS−PAGE and migrate as proteins of 140 and 20−26 kDa, respectively.

The expressed α_2/δ protein was analyzed by reducing and non-reducing SDS−PAGE (Figure 5a) and compared to the native α_2/δ protein from rabbit skeletal muscle t-tubules. Both the KT3 and α170 antibodies stained an expressed protein of ∼120 kDa under reducing conditions (Figure 5a, lanes 1 and 2). In addition, the α170 antibody stained an expressed protein of ∼140 kDa (Figure 5a, lane 2). Expressed proteins of ∼29−32 kDa were also stained by the α170 antibody (Figure 5b) however, staining of these proteins was inconsistent (not seen in lane 2 of Figure 5a). The native t-tubule α_2/δ subunit migrated as an α_2 peptide at ∼140 kDa and δ peptides at 20−26 kDa (Figure 5a, lane 3). Under non-reducing conditions the α170 antibody stained expressed proteins of ∼120 and ∼170 kDa (Figure 5a, lane 4) while the native t-tubule α_2/δ migrated at ∼170 kDa (Figure 5a, lane 5). (Nonspecific staining of a ∼195 kDa protein by α170 was observed under both reducing and non-reducing conditions, Figure 5a, lanes 2 and 4, and Figure 5b.) Similar results were obtained with another α_2-specific antibody (Figure 5c, lane 1). We observed that expression of the ∼120 kDa protein preceeded that of the ∼140 kDa protein (data not shown) and incubation of infected cells with tunicamycin, a widely used inhibitor of N-linked glycosylation, resulted in no detection of the ∼140 kDa protein and increased levels of the ∼120 kDa protein (Figure 5c, lanes 2 and 3). On the basis of these results the α_2/δ subunit appeared to be expressed as an ∼120 kDa "pro-form" (non-glycosylated, non-processed) and was processed to an ∼170 kDa disulfide-linked α_2/δ subunit which, under reducing conditions, dissociated to an ∼140 kDa α_2 peptide and δ peptides of 29−32 kDa. This corresponds well with the behavior of the native α_2/δ subunit. (Reasons for the lack of staining of the expressed δ peptides by the KT3 antibody and inconsistent staining by the α170 antibody are unclear but may be due to reduced immunoreactivity resulting from aberrant glycosylation of these peptides as suggested by molecular masses slightly higher than were expected.)

Co-Expression of L-Channel Subunits. In order to co-express the L-channel subunits, Sf9 cells were simultaneously infected with the desired combination of recombinant viruses. Western blot analysis of crude membrane fractions showed that Sf9 cells were able to co-express various combinations of channel subunit proteins (Figure 6a). Immunohistochemical analysis demonstrated co-expression of the subunit proteins in single cells (data not shown), although cells expressing only one subunit were also detected (data not shown).

Numerous studies in mammalian cells have established that co-expression of channel subunits can modulate the ability of the α_1 subunit to bind dihydropyridines (DHPs), a class of drugs that specifically target L-type Ca channels (*22*, *35*, *39*−*42*). We therefore performed DHP binding studies to obtain an indication that the expressed subunits formed functional complexes in the Sf9 cells. When expressed alone the α_{1C} subunit displayed no measurable binding of the radiolabeled DHP antagonist, [^{3}H]PN200-110, at concentrations of up to 10 nM in either membrane or whole cell binding assays. However, when α_{1C} was co-expressed with both the α_2/δ and β_{2a} subunits, [^{3}H]PN200-110 binding was restored with affinity nearly equivalent to that of native channels (*43*, *44*). Results from Scatchard analyses gave a K_d of 0.20 ± 0.06 nM ($n = 5$). The B_{max} values calculated for these five experiments were 40, 116, 154, 648, and 1240 fmol per milligram of whole-cell protein. The variability in B_{max} data may reflect differences in co-infection efficiency between experiments. Limited binding studies were also performed for the α_{1S} subunit. A low level of saturable DHP

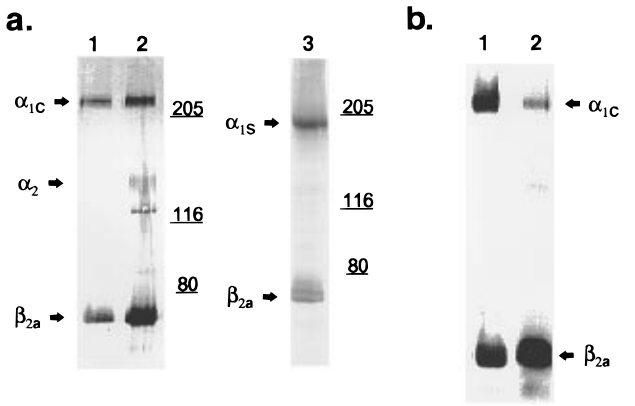

FIGURE 6: Co-expression of L-channel α_1, α_2/δ, and β_{2a} subunits in Sf9 cells. (a) Western blot analysis of membrane proteins from cells co-expressing Ca channel subunits. Shown is the α_{1C} subunit co-expressed with the β_{2a} subunit (lane 1) or both the α_2/δ and β_{2a} subunits (lane 2). Lanes 1 and 2 were simultaneously stained with the Card C, β_2, and alpha2 antibodies. Also shown is the α_{1S} subunit co-expressed with the β_{2a} subunit (lane 3). Lane 3 was simultaneously stained with the Sk N and β_2 antibodies. (b) Co-immunoprecipitation of co-expressed L-channel subunits. Lane 1, Western blot of crude membrane starting material prior to solubilization and immunoprecipitation showing co-expression of both α_{1C} and β_{2a} subunits. The Card C and β_2 antibodies were used for immunostaining. Shown is 1/10 (100 μg) of the total crude membrane starting material used for the co-immunoprecipitation assay. One mg of the crude membrane proteins shown in lane 1 was solubilized with 1% digitonin followed by immunoprecipitation with the β_{gen} Ab only. In lane 2, the entire immunoprecipitation pellet was analyzed by Western blot. Immunostaining with the Card C and β_2 antibodies shows presence of co-immunoprecipitated α_{1C} subunit in addition to directly precipitated β_{2a} subunit (lane 2). Since the β_{2a} subunit expressed at higher levels (see text for details), the immunoprecipitate likely contained β_{2a} subunit complexed with α_{1C} as well as free, noncomplexed β_{2a} as was suggested by the relative intensities of immunostaining by the Card C and β_2 antibodies in lane 2.

binding was observed when the α_{1S} subunit was expressed alone, and co-expression with the β_{2a} subunit resulted in a 10-fold increase in the B_{max} (data not shown). These results suggested that the co-expressed channel subunits interacted and were able to display properties of functional channel complexes in the Sf9 cell membranes.

Results from co-immunoprecipitation studies also supported this conclusion. The β_{gen} antibody was used to precipitate digitonin solubilized proteins from α_{1C} BV and β_{2a} BV infected Sf9 cells. Immunoblot analysis of the precipitated proteins with the Card C and β_2 antibodies showed that, in addition to the β_{2a} subunit protein, the α_{1C} subunit was also precipitated by the β_{gen} antibody (Figure 6b, lane 2). The β_{gen} antibody was unable to precipitate the expressed α_{1C} subunit without β_{2a} subunit co-expression (data not shown).

Phosphorylation of the α_{1C}, α_{1S}, and β_{2a} Subunits by Protein Kinases A and C. L-type channel α_1 and β subunits contain consensus sites for phosphorylation by the cAMP-dependent protein kinase (PKA) and/or protein kinase C (PKC) and thus represent potential targets through which regulation of channel function by phosphorylation may occur. In contrast, the "universal" α_2/δ subunit does not appear to be a target of phosphorylation. While the regulation of L-type channels by protein kinases has been extensively studied using electrophysiological techniques, very few biochemical studies have determined the ability of channel proteins to act as substrates for these enzymes, largely

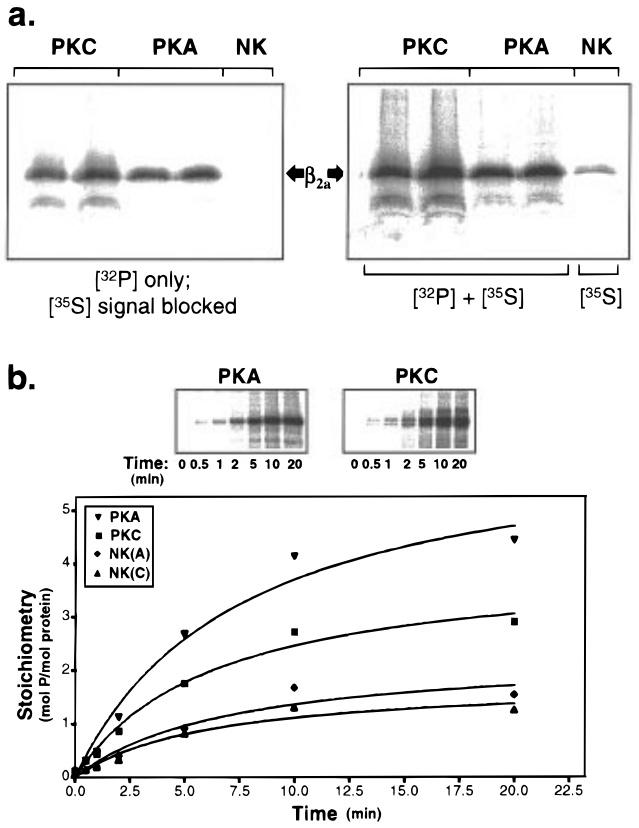

FIGURE 7: *In vitro* phosphorylation of expressed β_{2a} subunits by PKA and PKC. (a) Membranes were prepared from β_{2a} expressing Sf9 cells after metabolic labeling with [^{35}S]methionine and subsequently used in *in vitro* kinase assays with either PKA or PKC as indicated. Shown is a phosphorimage with (left) and without (right) blocking of ^{35}S-label (see text for details). The "no kinase" (NK) lane containing only ^{35}S-labeled membranes is shown as a control for the blocking procedure. (b) Time course of β_{2a} subunit phosphorylation *in vitro* by PKA (inverted triangles) and PKC (squares). Results from control (no kinase added) reactions are shown for both PKA (diamonds) and PKC (triangles). Inset, phosphorimage data used to generate time course curves.

because the α_{1C} and β_{2a} subunits have been extremely difficult to detect in native tissues. Previous studies, including two recent ones, have examined phosphorylation of the α_{1C} subunit *in vitro* by PKA; however, in each of these the α_{1C} protein was first immunopurified before being phosphorylated rather than being studied in its membrane-associated state, and, in one report, only the carboxy-terminus of the α_{1C} protein was studied after its expression as a fusion protein (*15*, *45*−*47*). In addition, little has been reported regarding α_{1C} as a substrate for PKC, and phosphorylation of the β_{2a} subunit is virtually unstudied. Lastly, several electrophysiological studies have suggested that subunit interactions may be necessary for certain phosphorylation reactions to occur, but this has not been directly investigated in biochemical studies (*48*−*52*). We investigated the ability of the expressed, membrane-associated α_{1C}, α_{1S}, and β_{2a} subunits to serve as substrates, alone or in combination, for PKA and PKC *in vitro*. (Note all phosphorylation reactions described here were performed *in vitro*.)

The β_{2a} subunit is predicted by sequence analysis to contain PKA and PKC phosphorylation sites and, indeed, when expressed alone was found to be an excellent substrate for PKA and PKC *in vitro* (Figure 7). The stoichiometry of phosphorylation of the β_{2a} subunit was determined using a dual-labeling method. After metabolic labeling of the

expressed β_{2a} subunit with [^{35}S]methionine, crude membrane preparations were phosphorylated *in vitro* with PKA or PKC. Since an ^{35}S signal can be easily blocked with a thin plastic sheet, it was possible to quantitate both the ^{35}S and ^{32}P signals from the β_{2a} subunit by phosphorimaging with and without blocking of the ^{35}S signal (Figure 7a). After conversion of ^{35}S and ^{32}P signals to moles of β_{2a} protein and moles of phosphate, respectively, the stoichiometry of phosphorylation was calculated. When no purified exogenous kinase was added to phosphorylation reactions that contained the supplementary reagents for either PKA or PKC phosphorylation, approximately one mole of phosphate per mole of protein was incorporated into the expressed β_{2a} subunit (Figure 7b) by endogenous Sf9 cell kinase(s) in the crude membrane preparation. Addition of PKA or PKC increased phosphorylation over the basal phosphorylation. The net increase in stoichiometry was ~2 mol of phosphate per mole of protein for PKA and 1−2 mol of phosphate per mole of protein for PKC. Time courses of phosphorylation showed that both kinases rapidly phosphorylated the expressed β_{2a} subunit (Figure 7b). PKC was observed to induce a mobility shift in the β_{2a} subunit (Figure 7b, inset).

The membrane-associated α_{1C} subunit, expressed alone, was revealed to be an excellent substrate for both PKA and PKC. Large increases in phosphorylation were observed when either kinase was added to phosphorylation reactions containing α_{1C} in Sf9 cell membranes compared to reactions with no added kinase (Figure 8a). The stoichiometry of phosphorylation of expressed α_{1C} by both PKA and PKC was determined using membrane preparations in which cell surface α_{1C} could be isolated as a consequence of biotinylation. Cell surface expressed α_{1C} was specifically separated and studied because we felt that it represented the most appropriately processed or "native-like" fraction of the expressed α_{1C} and because we wished to avoid studying α_{1C} that was being processed by either biosynthetic or degradation pathways. We reasoned that this approach would allow for accurate determination of stoichiometries of phosphorylation as the sites available for phosphorylation in the membrane-associated protein are most likely to be available physiologically. In particular, it was important to assess the phosphorylation potential of the membrane-localized α_{1C} and to compare results to studies in which sites might be exposed or occluded when studies are performed on immunoprecipitated proteins (*15, 45*−*47*). Using a Card C standard curve such as that shown in Figure 2b, the stoichiometries of phosphorylation for the expressed α_{1C} subunit were found to be 1 mol of phosphate per mole of protein after phosphorylation by PKA *in vitro* ($n = 2$) and 2−3 mol of phosphate per mole of protein after phosphorylation by PKC *in vitro* ($n = 3$). Standard curves relating moles of α_{1C} subunit protein to Card C antibody staining intensity were generated for each experiment to account for variability from experiment to experiment. The variability results from variability that arises in immunoprecipitation. Indeed the Western blots were performed to allow us to control for this variability in the quantitations. We assume that the diffuse nature of the bands in the phosphorimage relate to the diffuse migration of the α_1 proteins. This is seen with expressed α_1 subunits as well as with α_1 subunits from native tissue and may be due to post-translational modifications of the α_1 protein such as glycosylation. For the purposes of analysis, only phosphorimage signals directly corresponding to Western blot signals were used to determine the phosphorylation of a channel subunit protein by a purified kinase. In general, differences in phosphorimage signals are directly dependent on the amounts of protein present, the specific activity of the label used, and the exposure time of the phosphorimage. It is important to note that in all cases the phosphorylation observed when no kinase was added constituted a small percentage of the phosphorylation observed when purified kinases were added to reactions. Furthermore, when quantifying the responses the "no kinase added" phosphorylation was subtracted from the corresponding "kinase added" phosphorylation to give the phosphorylation solely attributable to that mediated by the added purified kinase. Phosphorylation of channel subunit proteins were compared after being converted to moles of phosphate per mole of protein thereby accounting for differences in amounts of protein, specific activity of the label, and phosphorimage exposure times from experiment to experiment. Given the controls used, the results were very reproducible.

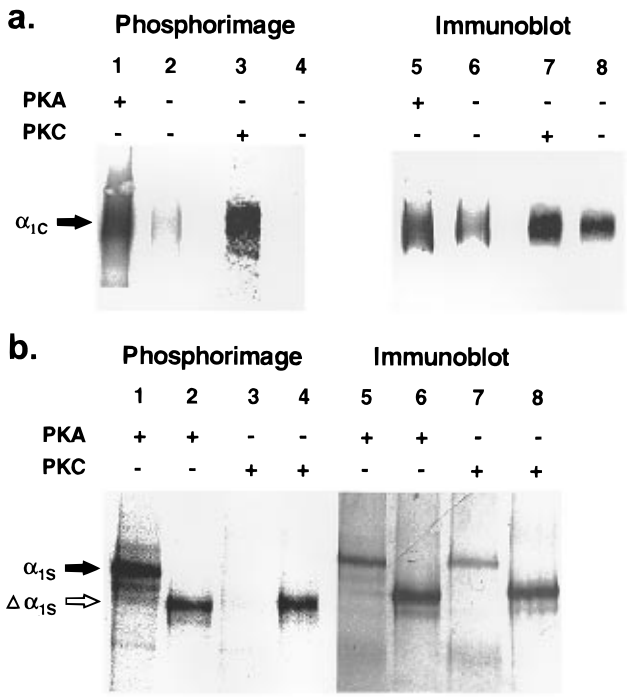

FIGURE 8: *In vitro* phosphorylation of α_{1C} and α_{1S} subunits by PKA and PKC. (a) Phosphorimage (lanes 1−4) and corresponding Western blot (lanes 5−8) of expressed α_{1C} subunit phosphorylated by PKA (lanes 1 and 5) or PKC (lanes 3 and 7). Results from control (no kinase added) assays are also shown (lanes 2, 4, 6, and 8). The Card C Ab was used for immunostaining. (b) Phosphorimage (lanes 1−4) and corresponding Western blot (lanes 5−8) of α_{1S} subunit phosphorylated by PKA (lanes 1, 2, 5, and 6) or PKC (lanes 3, 4, 7, and 8). Odd-numbered lanes contain expressed α_{1S} subunit while even-numbered lanes contain native α_{1S} subunit from rabbit skeletal muscle. Sk N Ab was used for immunostaining.

In contrast to the α_{1C} subunit, the α_{1S} subunit, when expressed alone, showed a slightly different and interesting pattern of phosphorylation in *in vitro* studies. The expressed α_{1S} was readily phosphorylated by PKA (Figure 8b, lane 1) and to a greater extent than the native carboxy-terminal truncated form of α_{1S} from skeletal muscle (Figure 8b, lane 2). To calculate the stoichiometries of phosphorylation, the amounts of expressed α_{1S} subunit protein were also determined using quantitative immunoblotting. The amount of native α_{1S} in skeletal muscle t-tubule membranes was quantified by DHP binding and these values were used to

generate standard curves relating antibody staining intensity to pmol of α_{1S} (data not shown). From this analysis it was determined that phosphorylation by PKA *in vitro* resulted in incorporation of 6−7 mol of phosphate per mole of Sf9 expressed α_{1S} subunit, while the native t-tubule α_{1S} incorporated 2−3 mol of phosphate per mole of protein. This result was expected since additional phosphorylation sites are predicted to be present in the carboxyl-terminus of the full-length expressed α_{1S} protein that are missing in the carboxy-terminal truncated protein present in the t-tubule membrane preparation (*16*, *34*). However, somewhat surprisingly, the α_{1S} subunit expressed alone failed to serve as a substrate for PKC (Figure 8b, lane 3) while the truncated α_{1S} from t-tubule was phosphorylated (Figure 8b, lane 4). This suggested that the full-length α_{1S} might contain sequences that inhibited phosphorylation by PKC and/or that other subunits might need to associate with α_{1S} in order for it to be a substrate for PKC (note that the native skeletal muscle α_{1S} is truncated and complexed with the α_2/δ and β_1 subunits). We addressed these possibilities below.

Phosphorylation of Co-Expressed L-Type Channel Subunits by Protein Kinases A and C. In order to directly test whether co-expression of subunits altered their ability to serve as substrates for PKA and PKC, we performed phosphorylation studies with the α_{1C} or α_{1S} subunits co-expressed with β_{2a} in parallel with studies of the α_1 subunits expressed alone. Phosphorylation of the α_{1C} subunit by both PKA and PKC was not changed upon co-expression with the β_{2a} subunit (Figure 9a); the stoichiometry of phosphorylation was identical to that obtained for α_{1C} alone. The α_{1S} subunit also remained an excellent substrate for PKA when co-expressed with the β_{2a} subunit in Sf9 cells with no change in the stoichiometry of phosphorylation (Figure 9b, lane 3). In contrast, the α_{1S} subunit became an excellent substrate for PKC *in vitro* when expressed with the β_{2a} subunit (Figure 9b, lane 1). The expressed α_{1S} subunit was phosphorylated to a stoichiometry of 2−3 mol of phosphate/mole of protein by PKC. This extent of phosphorylation was very similar to that obtained for the native truncated α_{1S} (which is associated with the native β_1 subunit) from skeletal muscle (Figure 9b, compare lanes 1 and 2). This result demonstrates a necessity for subunit interaction to occur in order to allow for PKC-mediated phosphorylation of α_{1S} whereas a similar interaction does not appear to be required for α_{1C} phosphorylation by PKC. In addition, the results suggest that the PKC sites are located proximal to the site of truncation in the α_{1S} carboxy-terminus as no additional sites for PKC phosphorylation are contained in the full-length versus the truncated versions of α_{1S}, in marked contrast to the situation for the sites of PKA phosphorylation in this subunit.

Finally, we investigated whether co-expression affected the ability of the β_{2a} subunit to serve as a substrate for PKA or PKC. When co-expressed with the α_{1C} subunit, the β_{2a} subunit remained an excellent substrate for both kinases (Figure 9c) and the stoichiometry of phosphorylation was identical to that obtained when β_{2a} was expressed alone.

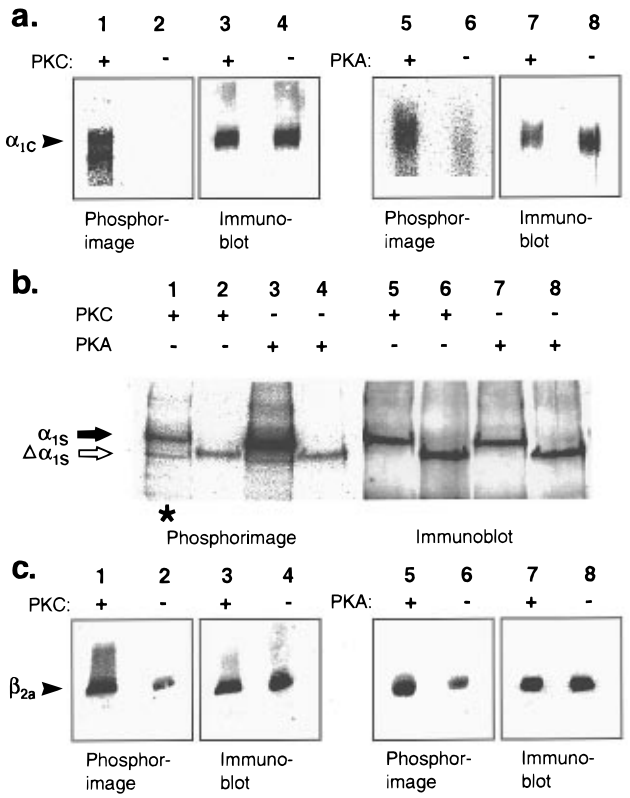

FIGURE 9: *In vitro* phosphorylation of co-expressed L-channel subunits by PKA and PKC. (a) Membrane proteins from Sf9 cells co-expressing α_{1C} and β_{2a} subunits were phosphorylated *in vitro* with either PKC (lanes 1 and 3) or PKA (lanes 5 and 7). Results from control (no kinase added) assays are also shown (lanes 2, 4, 6, and 8). The α_{1C} subunit was immunoprecipitated and analyzed by phosphorimaging (lanes 1, 2, 5, and 6) or Western blot (lanes 3, 4, 7, and 8). The Card C antibody was used for immunostaining. (b) Phosphorimage (lanes 1−4) and corresponding Western blot (lanes 5−8) of α_{1S} subunit phosphorylated by PKC (lanes 1, 2, 5, and 6) or PKA (lanes 3, 4, 7, and 8). Odd-numbered lanes show results from experiments using α_{1S} subunit co-expressed with β_{2a} subunit from Sf9 cells while even-numbered lanes contain native α_{1S} subunit from rabbit skeletal muscle. Asterisk highlights observed phosphorylation of α_{1S} by PKC upon co-expression with β_{2a} subunit (compare with Figure 8b). Sk N Ab was used for immunostaining. (c) Membrane proteins from Sf9 cells co-expressing α_{1C} and β_{2a} subunits were phosphorylated *in vitro* with either PKC (lanes 1 and 3), PKA (lanes 5 and 7) or no exogenous kinase (lanes 2, 4, 6, and 8). The β_{2a} subunit was immunoprecipitated, and analyzed by phosphorimaging (lanes 1, 2, 5, and 6) or Western blot (lanes 3, 4, 7, and 8). The β_2 antibody was used for immunostaining.

DISCUSSION

The development of methods to successfully express subunits of L-type Ca channels with recombinant baculovirus has allowed for new insights into the events associated with regulation of these channels by protein phosphorylation. In particular, we provide the first evidence that (i) the β_{2a} subunit can be stoichiometrically phosphorylated *in vitro* by both PKA and PKC, and by an endogenous membrane kinase in Sf9 cells; (ii) the membrane-bound α_{1C} subunit can be stoichiometrically phosphorylated at two or three sites *in vitro* by PKC; and (iii) phosphorylation of the α_{1S} subunit by PKC requires association with a β subunit. In addition, here we report the first characterization of phosphorylation of the full-length α_{1C} subunit by PKA while maintained in the native environment of the plasma membrane. A number of previous studies have detected phosphorylation of α_{1C} by PKA; however, in each case phosphorylation was performed after immunopurification of the protein (*15*, *45*−*47*). These studies did not address the potential concern that sites of phosphorylation are exposed as a result of solubilization with detergent, or occluded as a result of the interaction of α_{1C} with the precipitating antibody. While some information existed about the phosphorylation of the α_{1S} subunit from

native skeletal muscle membranes (*3*−*11*), nothing was known about the contribution of the other subunits to modify α_{1S} phosphorylation.

When purified from native tissues, a significant fraction of all L-type α_1 subunit isoforms have been shown to be, or are postulated to be, truncated at their carboxyl-termini and, as a consequence, lack one or more potential PKA and PKC phosphorylation sites (*12, 15, 16, 45*). Indeed, the majority of the native α_{1C} subunit purified from heart or brain migrated on SDS gels as a carboxy-terminal truncated protein of 195 kDa and failed to serve as a substrate for PKA (*12, 15, 45*). In contrast, for reasons that are not clear, biochemical analyses of heterologously expressed L-channel α_1 subunits have demonstrated expression of full-length proteins (*15, 22, 41, 42, 47, 53*). Given the possibility that the full-length α_1 subunits are components of functional channels in native tissues, and that the potential phosphorylation sites located in the carboxyl-terminus may play critical roles in channel regulation, we reasoned that the full-length α_1 subunits expressed by the Sf9 cells provided an important opportunity to characterize the phosphorylation of these normally rare and elusive membrane proteins. Recent studies have presented evidence suggesting Ser^{1928} in the carboxyl-terminus of the α_{1C} subunit to be a site of PKA phosphorylation (*46, 47, 54*). It is likely that this site corresponds to that which we have identified to be stoichiometrically phosphorylated *in vitro* in this study, since the truncated α_{1C} is not a substrate for PKA (*12, 15, 45*). PKC phosphorylated the α_{1C} subunit to a stoichiometry of 2−3 mol of phosphate per mole of protein. Several previous studies have suggested that PKC regulates channels that are predicted to contain the α_{1C} subunit (*45, 49, 51, 55*−*57*). Additionally, a recent study has implicated PKC phosphorylation of the neuronal α_{1B} subunit as an important regulatory mechanism in channel modulation by G-protein subunits (*58*). Identification of specific sites phosphorylated on the α_{1C} subunit by PKC will be an important focus of future studies.

The phosphorylation data shown here with the β_{2a} subunit are the first to clearly characterize this protein as a kinase substrate *in vitro*. Stoichiometric phosphorylation of this subunit by both PKA and PKC as well as the endogenous Sf9 cell kinase lends credence to the possibility that this subunit may play an important role in channel regulation (*48, 51, 52, 59*). Phosphorylation of the β subunit could be particularly critical if the α_{1C} subunit is truncated in native cells and lacks the phosphorylation sites located in its carboxyl-terminus. Future studies will focus on revealing the phosphorylation sites on the β subunit and their roles in channel regulation.

Several previous studies using electrophysiological approaches have suggested that combinations of channel subunits might be necessary for phosphorylation to occur (*48*−*52*). Here we present the first direct test of the ability of the channel subunits to act as kinase substrates *in vitro* when expressed alone and in combination. Of interest was the finding that the α_{1S} subunit could only serve as a substrate for PKC *in vitro* when co-expressed with the β_{2a} subunit, providing the first direct biochemical demonstration of a role for subunit interactions in modulation of channel phosphorylation. At this point we do not understand the requirement for the β subunit at the molecular level. It is possible that association with the β subunit allows the α_{1S} subunit to assume a conformation necessary to allow for recognition by PKC. When contrasted with studies of the α_{1C} subunit these results suggest that subunit interactions may play differential roles in the regulation of distinct channel isoforms by protein kinase mediated phosphorylation. Interestingly, in previous studies, we found that the PKC-mediated phosphorylation of the α_{1S} subunit in purified and reconstituted skeletal muscle channels (consisting of truncated α_{1S}, β_1, γ, and $\alpha_2\delta$) required a particular composition of lipids in the reconstitution mixture, while the PKC-mediated phosphorylation of the β subunit had less stringent requirements (*60*). Taken together with the present results, the data suggest that phosphorylation of the α_{1S} subunit may be conformation sensitive. The effect of the β subunit to modify phosphorylation is also interesting in light of the results of recent electrophysiological studies in which certain neuronal voltage-dependent calcium channels required β subunit co-expression for PKC modulation of channel function to occur (*52*). Also, in other recent studies, it was suggested that β subunits play an important role in the voltage- dependent facilitation of channel activity, a process in which PKA has been suggested to play a role (*50*). However, in none of these studies was phosphorylation of the channel proteins directly monitored. Thus, it was not known if the requirement for the β subunits was to allow for phosphorylation of α_1 subunits, or for other reasons. While further studies will be required to dissect out the important contributions of each subunit, the present results highlight the importance of monitoring the actual phosphorylation reactions in order to understand how subunit interactions may modulate channel regulation by protein kinases.

The α_{1C} and β_{2a} subunits each served as excellent substrates for PKA and PKC whether expressed alone or together, suggesting that phosphorylation of either subunit by these two kinases can occur independently. However, the data do not yet provide insights into the question of whether one or both subunits must be phosphorylated in order to achieve channel regulation. Our results support the hypothesis that regulation of α_{1C} containing (cardiac, smooth muscle, or brain) L-channels by protein kinases such as PKA and PKC occurs as a result of direct phosphorylation of either or both the α_{1C} and β_{2a} subunits.

The phosphorylation studies described here were made possible by the use of recombinant baculoviruses. Relatively modest levels of expression were achieved for the pore-forming α_1 subunits in Sf9 cells probably due to the fact that these are very large hydrophobic proteins. However the expression levels were in excess of those achieved in native tissues and, by using cells grown in suspension culture, we were able to produce protein amounts appropriate for biochemical studies. In contrast, Sf9 cells express high levels of multiple, membrane-localized isoforms of the β_{2a} subunit. Expression of the α_2/δ subunit in Sf9 cells and the observation of the non-glycosylated, non-processed, pro-form of the protein provides a unique opportunity to study the processing events involved in generation of the mature subunit protein. Our data suggests that glycosylation might be an important signal for the processing of the protein into the α_2 and δ peptides since the 120 kDa pro- form did not dissociate into the α_2 and δ peptides under reducing conditions (Figure 5a). Further studies will be necessary to ascertain the validity of this hypothesis. The role of this accessory subunit in channel function has been only sparsely characterized (*38, 40, 53*).

In the Sf9 cells, co-expression of the α_2/δ and β_{2a} subunits with the α_{1C} subunit restored high-affinity DHP binding to levels comparable to that of native α_{1C} containing L-type channels. This result was important since, together with results from immunoprecipitation studies in which co-expressed subunits were successfully co-immunoprecipitated, it suggested to us that Sf9 cells are able to appropriately assemble co-expressed subunits such that they are able to display properties of functional channel complexes.

In summary, we have shown that infection of Sf9 insect cells with recombinant baculoviruses is an excellent method to produce heterologously expressed L-channel subunits. This system has allowed for previously difficult, if not impossible, biochemical studies of phosphorylation of full-length channel subunits in native membrane environments by PKA and PKC *in vitro*. Future studies need to elucidate the functional role of phosphorylation of each subunit in channel regulation, further investigate the role of subunit interactions in regulation of channels by protein phosphorylation, and conclusively determine the status of the α_1 subunits as full-length or truncated proteins in native tissues. In the latter case, the expressed, full-length α_1 subunit proteins can be used to assess mechanisms of truncation of the carboxyl-terminus including screening experiments to identify the cellular protease(s) responsible for cleavage. This expression system should prove useful in the biochemical investigation of these and other important questions regarding the function and regulation of L-type Ca channels.

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