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Post-Translational Modifications of β Subunits of Voltage-Dependent Calcium Channels

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Different post-translational modifications of Ca channel β subunits have been identified. Recent studies have characterized the palmitoylation of the Ca channel β_{2a} subunit, as well as one effect of this modification on channel function. The potential importance of palmitoylation on other channel properties is discussed. Other studies have addressed the role of phosphorylation of β subunits in the regulation of voltage-dependent Ca channels. Phosphorylation of β subunits by second messenger-activated protein kinases, as well as by unidentified protein kinases, may affect interactions between channel subunits and other aspects of channel function. The differential modification of Ca channel β subunit isoforms by post-translational events likely results in diversely regulated channels with unique properties.

KEY WORDS: Calcium channels; post-translational modification; palmitoylation; phosphorylation.

The regulation of cellular proteins, including ion channels, often involves some form of post-translational modification. To date, many different types of post-translational modifications have been identified, some of which are reversible and some of which are irreversible. A broad spectrum of moieties, including lipids, sugars, inorganic molecules, and even peptides, can be enzymatically incorporated into, or removed from, specific protein sequences in a tightly regulated and often dynamic manner. Post-translational modifications also underlie events such as the covalent multimerization of proteins through disulfide linkages. Most post-translational modifications have functional consequences. Cellular enzymes involved in the post-translational modification of proteins are located throughout the cell, including within the nucleus, cytoplasm, and cellular structures such as the endoplasmic reticulum and Golgi complex.

Different post-translational modifications fulfill different functional requirements. For example, the addition to proteins of lipids such as prenyl groups is thought to increase the hydrophobicity of a protein and facilitate interaction with lipid membranes. The addition or removal of a phosphate group from a protein may result in conformational changes which alter protein function and/or activity. In certain instances, proteins can be functionally removed from cells by the sequential addition of several ubiquitin peptides and subsequent recognition by cellular degradation machinery. However, the function of some well-characterized post-translational modifications, such as glycosylation, remains unclear.

Phosphorylation, palmitoylation, ubiquitination, and O-linked glycosylation are among the modifications considered to be reversible (Hochstrasser, 1996; Mumby, 1997; Hart, 1997). The reversibility of these modifications derives in part from the labile nature of their linkages, which are readily hydrolyzed by specific enzymes. Clearly, reversible modifications allow for the dynamic regulation of a protein through the controlled addition and removal of a specific moiety. In contrast, modifications such as myristoylation, isoprenylation, N-linked gycosylation, and disulfide linkages are difficult to remove and considered permanent modifications for the cellular life of the protein.

Several important post-translational modifications of voltage-dependent Ca channels have already been identified. These channels are minimally com-

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posed of a pore-forming α_1 subunit and two accessory subunits, $\alpha_2\delta$ and β (De Waard *et al.*, 1996; Hosey *et al.*, 1996). Of the various different types of α_1 subunits that have been identified, it appears that several serve as targets for channel regulation by protein kinase A (PKA), protein kinase C (PKC), or Ca-dependent kinases (Hosey *et al.*, 1996; McDonald *et al.*, 1994). The α_2 and δ subunits are encoded by the same gene and appear to be a universal component of all identified Ca channels to date (Jay *et al.*, 1991; De Jongh *et al.*, 1990; Chang and Hosey, 1988; Witcher *et al.*, 1994; Scott *et al.*, 1996; Liu *et al.*, 1996). The product of the $\alpha_2\delta$ gene is post-translationally processed to create a membrane-spanning δ subunit which is disuifide-bonded to an extracellular, highly glycosylated α_2 subunit (De Jongh *et al.*, 1990; Gurnett *et al.*, 1996; Jay *et al.*, 1991). Glycosylation was found to be necessary for the modulation of Ca channels by $\alpha_2\delta$, although the molecular mechanisms underlying this phenomenon are still unclear (Gurnett *et al.*, 1996). The various β subunits that have been identified as components of different types of Ca channels also appear to be regulated by post-translational modifications (Leung *et al.*, 1988; Castellano and Perez-Reyes, 1994). This article will focus on modifications of Ca channel β subunits and the effects that these modifications may have on channel function.

MULTIPLE β SUBUNIT ISOFORMS EXIST

Ca channel β subunits are highly hydrophilic and are oriented on the cytosolic face of the plasma membrane (Castellano and Perez-Reyes, 1994). Their intracellular localization makes them an attractive target for many intracellular modifying enzymes and thus a likely site for the regulation of Ca channels through post-translational mechanisms. To date, researchers have identified four distinct β subunit isoforms in mammals, along with a large number of alternatively-spliced variants (Castellano and Perez-Reyes, 1994). The primary sequence of the four β isoforms reveals two highly-conserved central domains which display greater than 70% identity, bridged by distinct linker regions and flanked by N-terminal and C-terminal domains which are unique to each isoform (see Fig. 1) (Castellano and Perez-Reyes, 1994; De Waard *et al.*, 1996). There exists a high degree of homology in the conserved domains, even between phylogenetically distant species.

Biochemical analysis of voltage-dependent Ca channels in native tissues has proven difficult due to the rarity of these proteins. As a result, many studies on Ca channels have been performed in heterologous expression systems such as *Xenopus* oocytes and cultured mammalian cells. In addition to facilitating the relatively high-level expression of channel proteins, these systems also permit the selective expression of different subunit combinations and have facilitated studies on the roles of individual subunits in channel function. Additionally, the evaluation of different mutant channel subunits in heterologous expression systems has been extremely valuable in the structure–function analysis of different channel subunits.

Different β isoforms exhibit differential tissue expression patterns that are well conserved between species. For example, the β_3 isoform appears to be a major neuronal isoform in every species from which it has been cloned (Castellano and Perez-Reyes, 1994; De Waard *et al.*, 1996). Likewise, the β_2 isoform appears to be the predominant isoform found in cardiac tissue, although the identity of the human cardiac β subunit is still controversial (Castellano and Perez-Reyes, 1994). While individual cells have been shown to express multiple types of voltage-dependent Ca channels, it is still unknown whether a single cell expresses more than one β subunit isoform. Additionally, different isoforms also exhibit multiple splice variants which can be expressed in a tissue-specific manner (Hullin *et al.*, 1992). An appealing theory is that the pairing of different α_1 subunits with different β subunits allows cells to generate heterogeneous channel complexes with diverse properties.

Despite the conserved distribution of types of β subunits in native tissues, studies of heterologously expressed channels, as well as detailed analyses of one major interaction site between the α_1 and β subunits, have not revealed any clear specificities in the pairing of different α_1 and β subunits (De Waard *et al.*, 1994, 1995, 1996; Pragnell *et al.*, 1994). In heterologous expression systems, the different β subunit isoforms have quantitatively and qualitatively similar effects on increasing Ca channel currents and shifting the voltage dependence on activation. Different β isoforms do exhibit some differences in properties such as inactivation kinetics (see below), although it remains unclear why a certain tissue would prefer a certain β isoform to another. One likely explanation is that the β subunit isoforms are regulated by differential modifications on their unique N-terminal and C-terminal regions. Accordingly, different combinations of α_1 and β iso-

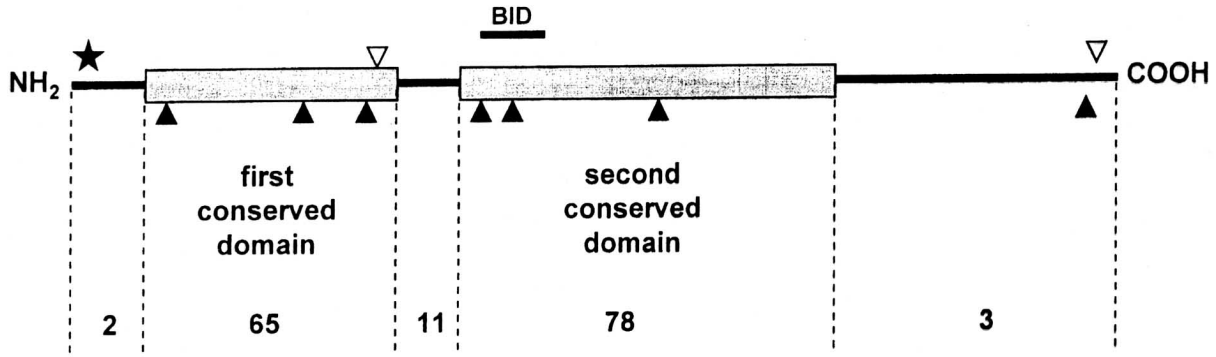

Fig. 1. The β_{2a} subunit is linearly represented to show the presence of two highly conserved regions, linked by a small linker domain and flanked by unique N-terminal and C-terminal regions. The numbers below represent the amino acid identity in each region between the four known β subunit isoforms (De Waard *et al.*, 1996); note the striking lack of amino acid identity in the N-terminal and C-terminal regions. Symbols indicate the sites of palmitoylation (★) as well as putative consensus sites for phosphorylation of both PKA (▽) and PKC (▲). Also shown above is the β interaction domain (BID), a region shown to be involved in interactions with the α_1 subunit (De Waard *et al.*, 1994).

forms could result in heterogeneous channels susceptible to differential regulation through each subunit. This hypothesis is supported by recent biochemical studies of the β subunit isoforms in heterologous expression systems, which identified post-translational modifications unique to the cardiac β_{2a} isoform (Chien *et al.*, 1995, 1996). Although several different β_2 splice variants have been identified to date, electrophysiological and biochemical studies have focused on the rat β_{2a} subunit, which was the first cardiac β isoform to be cloned (Perez-Reyes *et al.*, 1992). It should be noted that studies on post-translational modifications of β_{2a} discussed in this review may be specific to either this specific isoform or this particular species, as the universality of these modifications has not been extensively studied.

THE β_{2a} SUBUNIT IS POST-TRANSLATIONALLY MODIFIED

Surprisingly, immunohistochemical staining of human embryonic kidney (HEK) cells expressing the β_{2a} subunit revealed that this highly hydrophilic protein was localized to the plasma membrane, even in the absence of a co-expressed α_1 subunit (Chien *et al.*, 1995). Additionally, it was demonstrated that expression of the β_{2a} subunit facilitated the targeting of functional channel complexes to the plasma membrane (Chien *et al.*, 1995). When the β_{2a} protein was visualized on SDS-PAGE, it exhibited at least three distinct immunoreactive bands with relative electrophoretic mobilities ranging from the predicted 68 kDa size up to 72 kDa (Chien *et al.*, 1995). Pulse-chase analysis showed that the higher molecular mass isoforms were the result of sequential post-translational modifications, which occurred within four hours after synthesis of the nascent peptide (Chien *et al.*, 1995). The 68-kDa isoform appeared to be modified first to a 69-kDa isoform and subsequently to the 72-kDa isoform. These modifications of the β_{2a} subunit were also seen upon co-expression with the cardiac α_{1C} subunit (Chien *et al.*, 1995).

Initially, it was hypothesized that the post-translational modification may have been related to the unexpected localization of β_{2a} to the plasma membrane and/or the chaperone-like role of β_{2a} in targeting channels to the membrane (Chien *et al.*, 1995). The sequence of the β_{2a} subunit contained no predicted membrane-spanning regions, nor did it contain any of the consensus sequences for some modifications known to target hydrophilic proteins to cellular membranes, such as myristoylation and prenylation (Perez-Reyes *et al.*, 1992).

THE β_{2a} SUBUNIT IS PALMITOYLATED

Palmitoylation involves the covalent attachment of a 16-carbon fatty acid chain to cysteine residues through a thioester linkage (Mumby, 1997). The dynamic nature of this modification results from the labile nature of the thioester bond, which is readily hydrolyzed. Despite the large number of palmitoylated proteins identified, the exact roles of palmitoylation remain unclear, although evidence suggests that palmi-

toylation facilitates the membrane localization of certain proteins. There have been reports of proteins being depalmitoylated in a receptor-dependent manner and several groups have recently identified protein palmitoyl transferases, enzymes which preferentially attach and/or remove 16-carbon fatty acid chains to proteins (Mumby, 1997). Although sites of palmitoylation have been identified in many proteins, there is still no "consensus" sequence for predicting the modification of proteins by palmitoylation.

Metabolic labeling studies demonstrated that the β_{2a} subunit was palmitoylated when expressed in either HEK cells or Sf9 insect cells (Chien *et al.*, 1996). None of the other β subunit isoforms tested (β_{1b}, β_3, and β_4) were palmitoylated in similar metabolic labeling experiments, making palmitoylation the first known modification unique to a specific β isoform (Chien *et al.*, 1996). Site-directed mutagenesis was used to identify the sites of palmitoylation on β_{2a}, which were found to be Cys3 and Cys4 in the N-terminus (Chien *et al.*, 1996). Mutation of either of these two sites to Ser abolished palmitoylation, suggesting that they were both critical determinants of palmitoylation (Chien *et al.*, 1996). As expected, these sites were only found in the β_{2a} sequence. Replacement of the N-terminal regions of either β_{1b} or β_3 with the unique 16 amino acid N-terminus of β_{2a} led to palmitoylation of the resulting chimeric β subunits; palmitoylation of chimeric $\beta_{2a/1b}$ or $\beta_{2a/3}$ subunits did not occur when the β_{2a} N-terminus contained mutations at Cys3 and Cys4, confirming the importance of these residues as structural determinants of palmitoylation (Chien *et al.*, 1998).

Unexpectedly, mutation of the palmitoylation sites at Cys3 and Cys4 resulted in a loss of the higher molecular weight β_{2a} isoforms seen on SDS-PAGE (Chien *et al.*, 1996). This finding suggested that palmitoylation was required to allow for the post-translational modification that causes the apparent increase in molecular mass (see Fig. 2). However, palmitoylation per se did not appear to be responsible for the change in mobility of β_{2a} observed on SDS-PAGE. The evidence suggesting this conclusion is that: (1) while the β_{2a} protein could be solubilized from membranes with either detergent or salt, palmitoylated β_{2a} was observed only in detergent-solubilized fractions (Fig. 2, step 3), and (2) the higher molecular weight isoforms were seen in salt-soluble fractions but did not contain palmitate (Fig. 2, step 4). Thus, the addition of palmitic acid was itself not responsible for the apparent increase in the molecular weight of β_{2a}, but appeared to be necessary for a subsequent modification (Chien *et al.*, 1996). Additionally, these results suggested that palmitate was removed from the higher molecular weight β_{2a} protein, consistent with the reversible nature of this modification (Fig. 2, step 5).

So what is the post-translational modification that results in the higher molecular weight isoforms of β_{2a}? The answer is unclear, although anecdotal evidence has suggested that it may be phosphorylation. *In vitro* phosphorylation of bacterially-expressed β_{2a}, which does not exhibit multiple electrophoretic bands, resulted in higher molecular weight isoforms which were similar in size and electrophoretic mobility to those observed for mammalian cell-expressed β_{2a} (Gerhardstein and Hosey, unpublished observations). A progressive loss of higher molecular weight isoforms was observed upon incubation of membranes from mammalian cells expressing β_{2a} at 30°C; this loss was partially prevented by fluoride and high phosphate, which inhibit a variety of enzymes including phosphatases (Chien and Hosey, unpublished observation). Additionally, we have observed that the β_{2a} subunit heterologously expressed in mammalian cells exhibits a high stoichiometry of basal phosphorylation (Gao et al., 1997b). Further studies will hopefully confirm the identity of this modification as well as its potential importance to channel function.

FUNCTIONAL EFFECTS OF β_{2a} PALMITOYLATION

Surprisingly, the palmitoylation-deficient β_{2a} subunit fractionated with crude membrane particulate fractions, so it did not appear initially that palmitoylation was the mechanism responsible for the membrane localization of β_{2a} (Chien *et al.*, 1996). Consistent with this conclusion was the observation that the other three known β subunit isoforms, which were not palmitoylated, also fractionated predominantly with membrane particulate fractions (Chien *et al.*, 1996). However, subsequent analysis by confocal immunofluorescence microscopy showed that the palmitoylation-deficient β_{2a} mutant exhibited a much different pattern of staining compared to the wild-type β_{2a} subunit. Whereas the wild-type β_{2a} localized clearly to the plasma membrane, the palmitoylation-deficient mutant exhibited punctate intracellular staining (Chien *et al.*, 1998). The basis of this change in localization is under investigation and most certainly involves the interaction of the

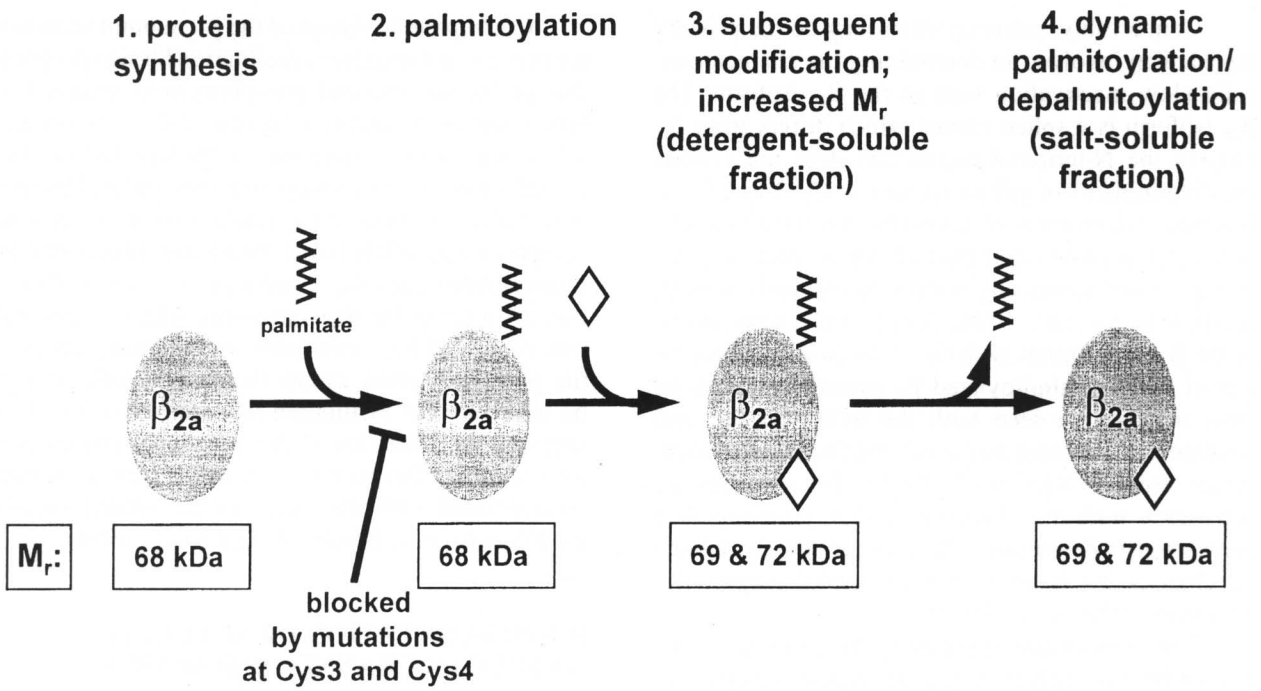

Fig. 2. This figure illustrates a model for the post-translational modification of the β_{2a} subunit. Following synthesis (step 1), the β_{2a} subunit is initially palmitoylated (step 2). Palmitoylation facilitates a subsequent modification (◇), which results in the higher molecular weight isoforms of β_{2a} seen on SDS-PAGE (step 3); this population of β_{2a} is seen in detergent-soluble fractions of β_{2a}. Subsequently, the palmitate group may be removed, while the other post-translational modification is retained (step 4); this population is observed as the salt-soluble fraction of β_{2a}. The palmitoylation-deficient mutants exhibit the lack of further modification as well as the lack of higher molecular weight isoforms. This model accounts for the presence of palmitoylated β_{2a} in detergent-soluble fractions, as well as the presence of palmitate-free higher molecular weight isoforms seen in salt-soluble fractions, and indicates that palmitoylation is itself not responsible for the shift in molecular weight seen on SDS-PAGE.

palmitoylation-deficient β_{2a} mutant with a different population of proteins than the wild-type β_{2a}.

When cells expressing the cardiac α_{1C} subunit and either the wild-type β_{2a} or the palmitoylation-deficient β_{2a}(Cys3,4Ser) mutant were studied using whole-cell patch-clamp analysis, a very intriguing result was found. Like the wild-type β_{2a} subunit, the palmitoylation-deficient mutant still targeted functional channels to the membrane, as measured by increases in whole-cell charge movement (Chien *et al.*, 1996). However, cells expressing the cardiac α_{1C} with the palmitoylation-deficient β_{2a} mutant exhibited much less current per amount of charge movement (Chien *et al.*, 1996). This decrease in whole-cell ionic current likely reflects a decrease in the mean single channel open probability and/or mean open time. It is not certain whether the lack of palmitoylation itself or lack of the subsequent post-translational modification responsible for the molecular weight shift is the cause of observed changes in channel function.

POTENTIAL ROLES OF β_{2a} PALMITOYLATION ON INACTIVATION AND FACILITATION

In heterologous expression systems, the β_{2a} subunit appears to slow Ca current inactivation significantly more than any other β subunit isoform. Studies involving chimeric β subunits demonstrated that the N-terminal region of the β_{2a} subunit was an important determinant of Ca channel inactivation (Olcese *et al.*, 1994). Replacement of the N-terminus of the β_{1b} subunit with the small 16 amino acid N-terminus of β_{2a} was sufficient to confer the slow inactivation property of the β_{2a} subunit upon co-expression with the α_{1E} subunit (Olcese *et al.*, 1994). This 16-amino acid region, which contains the sites identified for palmitoylation of the β_{2a} subunit, was also sufficient to confer palmitoylation upon the normally nonpalmitoylated β_{1b} and β_3 subunits (Chien *et al.*, 1998).

Conceivably, palmitoylation may be the primary mechanism involved in determining the characteristically slow inactivation seen in the β_{2a} isoform. The β_{2b} isoform is a splice variant which differs from β_{2a} only in the N-terminal region and does not contain the determinants of palmitoylation at Cys3 and Cys4. Notably, inactivation of channels containing the β_{2b} isoform was more rapid than channels containing β_{2a} and appeared kinetically similar to channels with β_3 and β_4 (Olcese *et al.*, 1994). Additionally, replacement of the β_{2a} N-terminus with the 15 amino acid N-terminus of the nonpalmitoylated β_3 subunit removed the slow inactivation seen with the wild-type β_{2a}, and resulted in a β subunit that conferred fast β_3-like inactivation kinetics (Qin *et al.*, 1996). These results are consistent with the hypothesis that palmitoylation plays a role in determining the effects of the β_{2a} subunit on channel inactivation. Recent results support this possibility (Qin *et al.*, 1998).

The reversibility of palmitoylation introduces the possibility that effects of β_{2a} on channel inactivation are regulated dynamically, potentially through a signal transduction pathway (Mumby, 1997). The addition of a hydrophobic palmitate group may anchor the β_{2a} N-terminus more tightly to the lipid bilayer, conceivably restricting certain protein conformations. Alternatively, the addition or removal of palmitate from the N-terminus may unmask the effects of other regions in the β_{2a} subunit which have been shown to be secondary regulators of inactivation (Qin *et al.*, 1996). Further studies using chimeric β subunits may further clarify the potential role of palmitoylation in channel inactivation, as well as the mechanism by which it may be regulated.

Another interesting electrophysiological finding involves the voltage-dependent facilitation of neuronal L-type Ca channels (Bourinet *et al.*, 1994). Facilitation of Ca currents by positive pre-pulses in *Xenopus* oocytes expressing the neuronal α_{1C} channel appeared to involve PKA and be independent of G proteins or intracellular calcium (Bourinet *et al.*, 1994). Facilitation was seen only in the presence of a β subunit. However, while the β_1, β_3, and β_4 subunits were permissive for facilitation, no facilitation was seen upon co-expression with the β_{2a} subunit (Cens *et al.*, 1996). Additionally, rat ventricular myocytes, which express the β_{2a} isoform, do not exhibit any pre-pulse induced facilitation, consistent with the hypothesis that some property of the β_{2a} subunit is inhibitory for facilitation (Cens *et al.*, 1996).

Could palmitoylation of the β_{2a} subunit somehow prevent pre-pulse induced facilitation? It was proposed that pre-pulses induced phosphorylated neuronal L-type channels to attain a "permissive" conformation which was more susceptible to opening and resulted in facilitation of currents upon the test pulse. Conceivably, palmitoylation of β_{2a} could anchor the channel complex more tightly to the membrane, preventing the channel from attaining a more permissive conformation upon pre-pulse depolarization. Alternatively, palmitoylation of β_{2a} could have an inhibitory effect on the phosphorylation events that are hypothesized to be necessary for facilitation to occur. A recent study supports the idea that if the functional requirement for a specific "facilitation-resistant" β subunit isoform corresponded with the only known subunit-specific modification of a β subunit (Qin *et al.*, 1998).

PHOSPHORYLATION OF SKELETAL MUSCLE Ca CHANNEL β SUBUNITS

Fairly extensive biochemical studies have been performed on L-type Ca channel subunits from skeletal muscle, as the relative abundance of L-type channels in the transverse tubule membranes has facilitated characterization of the native channel proteins in both intact cells and reconstituted systems (Hosey *et al.*, 1996). Skeletal muscle Ca channels contain the β_{1a} isoform, which was demonstrated to be a substrate *in vitro* for PKA and PKC (Hosey *et al.*, 1996). Treatment of newborn chick skeletal myocytes with the β-adrenergic agonist isoproterenol increased phosphorylation of the α_{1S} subunit and regulated channel activity, but did not result in phosphorylation of the β_{1a} subunit (see Fig. 3), suggesting that the β_{1a} subunit was less critical for regulation of these channels by PKA (Mundina-Weilenmann *et al.*, 1991). Likewise, experiments using channels reconstituted into liposomes showed that decreases in channel activity upon treatment with protein phosphatase 1c (PP-1c) correlated more strongly with dephosphorylation of the α_{1S} subunit rather than the β_{1a} subunit (Zhao *et al.*, 1994).

Reconstitution studies were also used to address the role of subunit phosphorylation in the regulation of skeletal muscle channels by PKC (see Fig. 3). Upon reconstitution of channels into artificial liposomes, the β_{1a} subunit was stoichiometrically phosphorylated by PKC, while the α_{1S} subunit was not a good substrate (Gutierrez *et al.*, 1994). However, under these conditions PKC treatment had no effect on channel activity

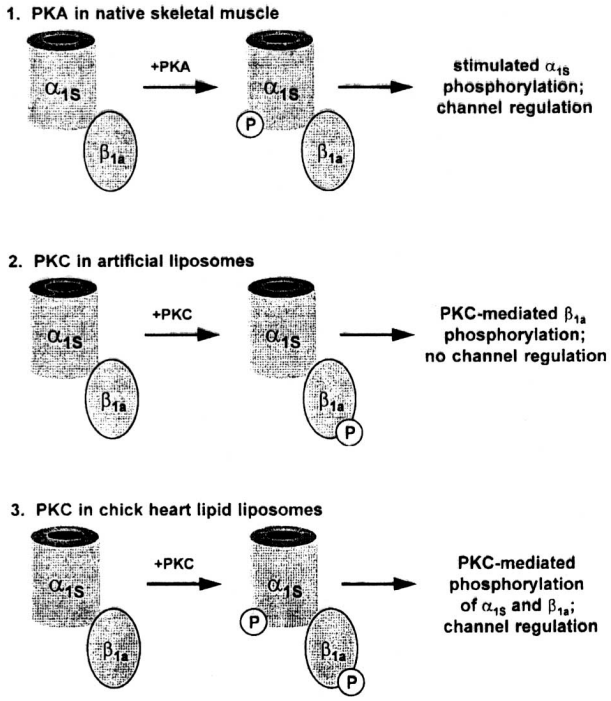

Fig. 3. Corresponding biochemical and functional events are summarized above in this figure on the regulation of skeletal muscle L-type Ca channels by both PKA- and PKC-mediated phosphorylation. In native myocytes (1), stimulation of the PKA pathway by isoproterenol increases channel activity and phosphorylation of the α_{1S} subunit with no effect on the β_{1a} subunit, suggesting that phosphorylation of the β_{1a} subunit is not necessary for regulation of these channels by PKA. In studies of channels reconstituted into artificial liposomes (2), stimulated phosphorylation of the β_{1a} by PKC was seen, but this phosphorylation did not result in any increases in channel activity. However, when channels were reconstituted into chick heart lipid liposomes (3), phosphorylation of both β_{1a} and α_{1S} resulted in increased channel activity, implying that phosphorylation of the α_{1S} protein was critical for the regulation of these channels by PKC.

in the artificial liposomes, implying that phosphorylation of the β_{1a} subunit was by itself insufficient to increase channel activity. By contrast, channels reconstituted into liposomes formed from chick heart lipids exhibited enhanced channel activity upon treatment with PKC, as well as stoichiometric phosphorylation of both the α_{1S} and β_{1a} subunits (Gutierrez *et al.*, 1994). These results implicated a critical role for phosphorylation of the α_{1S} subunit in channel regulation by PKC. While phosphorylation of the β_{1a} subunit alone appears inadequate for channel regulation, it is still unclear whether phosphorylation of β_{1a} is required in addition to phosphorylation of β_{1S} to allow for PKA- or PKC-mediated regulation of skeletal muscle channels.

Recent studies in Sf9 insect cells have suggested that subunit interactions involving β subunits play an important role in the regulation of skeletal muscle channels by phosphorylation. When the α_{1S} subunit was expressed alone in Sf9 cells, it was observed to be a substrate for PKA but not for PKC (Puri *et al.*, 1997). However, upon co-expression with the β_{2a} subunit, the α_{1S} subunit became a good substrate for PKC (Puri *et al.*, 1997). These results suggested that interaction with the β subunit may be an important determinant of channel regulation by PKC, perhaps by allowing α_{1S} to adopt a conformation permissive to phosphorylation by the kinase.

PHOSPHORYLATION OF CARDIAC Ca CHANNEL β SUBUNITS

Perhaps one of the best-studied systems for the regulation of an ion channel by phosphorylation is the cardiac L-type channel (Hosey *et al.*, 1996). These channels are formed minimally by the cardiac α_{1C} subunit, $\alpha_2\delta$, and the β_2 subunit (Hosey *et al.*, 1996; Gao *et al.*, 1997a). In native myocytes, stimulation of PKA through the β-adrenergic receptor pathway causes a significant increase in Ca currents (see McDonald *et al.*, 1994). Unlike the case with skeletal muscle, the biochemical events underlying this stimulation have been much more difficult to study due to the rarity of the cardiac L-type channel in native tissue. Furthermore, the unusual lability of the cardiac L-type channel complex has also hindered its purification, making it difficult to correlate functional events with biochemical findings. Nevertheless, some preliminary studies have been performed using channels from native myocytes. Studies from one group have implicated phosphorylation of the cardiac β subunit by PKA in the β-adrenergic receptor-mediated stimulation of canine myocardium (Haase *et al.*, 1993).

The sequence of the rat β_{2a} subunit contains several consensus sites for phosphorylation by PKA and PKC (Perez-Reyes *et al.*, 1992). The β_{2a} subunit expressed in bacteria, Sf9 cells, and transfected HEK cells is a good substrate for both PKA and PKC *in vitro* (Puri *et al.*, 1997). Little is known about the phosphorylation state of channel subunits in intact cells following activation of PKC, in part due to the fact that the functional effects of PKC on native cells remain controversial. Biochemical studies have instead focused on the stimulation of cardiac L-type channels

by PKA-mediated phosphorylation that has been well characterized from electrophysiological studies.

Recent studies have demonstrated a critical role for a class of kinase anchoring proteins known as AKAPs (*A Kinase Anchoring Proteins*) in the regulation of cardiac L-type Ca currents in transfected HEK cells (Gao *et al.*, 1997b). Phosphorylation of the α_{1C} subunit and stimulation of Ca currents upon activation of PKA required the presence of a functional AKAP (Fig. 4). By contrast, PKA-dependent increases in β_{2a} phosphorylation were observed independent of the presence of a functional AKAP, perhaps due to the more hydrophilic nature of the β_{2a} protein and a greater accessibility to cytosolic PKA (Gao *et al.*, 1997b). However, the results suggested that phosphorylation of the β_{2a} subunit alone was insufficient to confer channel stimulation by PKA. AKAPs have been reported to also be important for the voltage-dependent facilitation of skeletal muscle L-type Ca channels, although the target of this presumed phosphorylation event has not been identified (Johnson *et al.*, 1994, 1997).

Site-directed mutagenesis studies suggested that Ser1928 of the α_{1C} subunit was critical for regulation of the expressed cardiac channel by PKA, since this mutation abolished both the phosphorylation of the α_{1C} subunit as well as the channel stimulation observed upon activation of PKA in intact cells (Gao *et al.*, 1997b). Unexpectedly, another effect of the α_{1C} Ser1928 mutation was an increase in the extent of basal phosphorylation of the β_{2a} subunit. Additionally, the β_{2a} subunit co-expressed with the α_{1C} Ser1928 mutant no longer exhibited any increase in phosphorylation upon activation of PKA, perhaps because it was already fully phosphorylated in the basal state (Gao *et al.*, 1997b).

The importance of basal phosphorylation of β_{2a} is still unclear, and ongoing studies may further define its role in the regulation of channel function. Previous studies on the regulation of cardiac L-type channels had implicated a high level of basal channel phosphorylation in heterologous expression systems, accounting for some of the failures to reproduce the PKA-mediated regulation of channels seen in native cells (Perets *et al.*, 1996; Perez-Reyes *et al.*, 1994; Zong *et al.*, 1995). Basal phosphorylation of channel subunits may occur as early as during synthesis and multimerization of the different channel subunits in the endoplasmic reticulum. Alternatively, basal phosphorylation may occur following the targeting of channel complexes to the plasma membrane, regulating the interaction of the

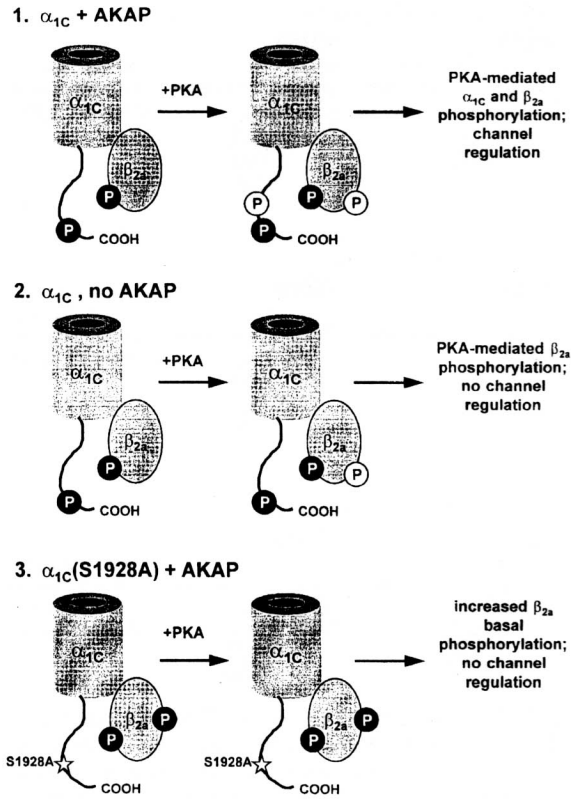

Fig. 4. Evidence from studies involving cardiac L-type channels heterologously expressed in mammalian cells has suggested that phosphorylation of the β_{2a} subunit is insufficient for PKA-mediated channel regulation. In this figure, basal phosphorylation is denoted by black circles (●) while PKA-mediated phosphorylation is denoted by white circles (○). In the presence of a functional AKAP protein (1), PKA phosphorylated both the α_{1C} and β_{2a} subunits, resulting in increased channel activity. In the absence of a functional AKAP and consequently the lack of α_{1C} phosphorylation (2), no channel regulation was seen despite stimulation of β_{2a} phosphorylation. When phosphorylation of α_{1C} was prevented by a mutation at Ser1928 (3), no channel regulation was seen, but increases in β_{2a} basal phosphorylation were observed. These findings suggested that phosphorylation of β_{2a} was by itself insufficient for conferring PKA-mediated regulation of the cardiac L-type channel. However, these results do not rule out a need for β_{2a} phosphorylation in addition to α_{1C} phosphorylation. Furthermore, these studies may not take into account the status of the α_{1C} C-terminus and its effect on the regulation of channels by β_{2a} phosphorylation in native myocytes (see text).

channel with a specific subset of proteins. Regardless, the presence of significant levels of basal phosphorylation of the β_{2a} subunit as well as the α_{1C} subunit suggests an important role for basal phosphorylation in channel function which should be clarified with further studies (Gao *et al.*, 1997b).

It remains unclear why mutations in the α_{1C} subunit would increase the basal phosphorylation of the

β_{2a} subunit, although these findings implicate some perturbation of subunit interactions. Potentially, the loss of phosphorylation in the α_{1C} Ser1928 mutant may result in a conformational change of the α_{1C} subunit, altering interaction with the β_{2a} subunit and allowing the β_{2a} subunit to become more readily phosphorylated by a basal kinase. Alternatively, Ser1928 of the α_{1C} subunit may be directly involved in interaction with the β_{2a} subunit, resulting in a secondary interaction site similar to that recently identified in the α_{1E} subunit (Tareilus *et al.*, 1997). Disruption of this interaction in the α_{1C} Ser1928 mutant may alter the conformation of the β_{2a} subunit and consequently affect the susceptibility of β_{2a} to phosphorylation by certain kinases. Regardless, these results suggest the potential importance of subunit interactions in regulating the state of β_{2a} subunit phosphorylation.

Although there is little direct evidence from heterologous expression systems that phosphorylation of the β_{2a} subunit is involved in regulation of cardiac L-type currents by PKA, detailed investigations on the role of the β_2 subunit in native myocytes have not yet been possible. Conceivably, changes in the C-terminal region of the α_{1C} protein may influence either the basal phosphorylation or the PKA-mediated phosphorylation of the β_{2a} subunit, as suggested by the studies involving β_{2a} and the α_{1C} Ser1928 mutant in the HEK expression system. Curiously, the C-terminus of the α_{1C} subunit is apparently truncated in partially purified L-type channels from various species (Hosey *et al.*, 1996; Gao *et al.*, 1997a). Recent immunohistochemical binding experiments in native rabbit ventricular myocytes have indicated that an intact α_{1C} C-terminal region is present in native myocytes (Gao *et al.*, 1997a). However, a co-translational or post-translational event involving regulated proteolysis of the α_{1C} C-terminus cannot be ruled out, and has been demonstrated for L-type channels in other tissues (Hell *et al.*, 1996). It is conceivable that the C-terminus of the α_{1C} subunit is proteolytically processed in myocytes but remains functionally associated with the L-type channel complex and able to influence subunit interactions. Future studies will hopefully provide answers to some of the issues raised herein regarding post-translational modifications of channel subunits and their roles in both channel maturation and function.

CONCLUSIONS

While the development of heterologous expression systems has facilitated the identification of different post-translational modifications on Ca channel subunits, much remains to be learned about the effects of these modifications on channel function in native cells. The regulation of modifications such as palmitoylation and basal phosphorylation remains unclear. Modifications of different β subunits, as well as the pathways involved in regulating these modifications, could also vary depending on cell type. The interaction of β subunits with different types of α_1 subunits could also affect the post-translational modifications of both types of subunits, potentially allowing further heterogeneity in the regulation of Ca channels. The importance of different subunit modifications in the regulation of Ca channels may be better understood as more is known regarding factors such as subunit interactions and the status of channel subunits in native cells.

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REFERENCES

Bourinet, E., Charnet, P., Tomlinson, W. J., Stea, A., Snutch, T. P., and Nargeot, J. (1994). *EMBO J.* **13**, 5032–5039.

Castellano, A., and Perez-Reyes, E. (1994). *Biochem. Soc. Trans.* **22**, 483–488.

Cens, T., Mangoni, M. E., Richard, S., Nargeot, J., and Charnet, P. (1996). *Pflugers Arch.— Eur. J. Physiol.* **431**, 771–774.

Chang, F. C., and Hosey, M. M. (1988). *J. Biol. Chem.* **263**, 18929–18937.

Chien, A. J., Zhao, X., Shirokov, R. E., Puri, T. S., Chang, C. F., Sun, D., Rios, E., and Hosey, M. M. (1995). *J. Biol. Chem.* **270**, 30036–30044.

Chien, A. J., Carr, K. M., Shirokov, R. E., Rios, E., and Hosey, M. M. (1996). *J. Biol. Chem.* **271**, 26465–26468.

Chien, A. J., Gao, T., Perez-Reyes, E., and Hosey, M. M. (1998). *J. Biol. Chem.* **273**, in press.

De Jongh, K. S., Warner, C., and Catterall, W. A. (1990). *J. Biol. Chem.* **265**, 14738–14741.

De Waard, M., Pragnell, M., and Campbell, K. P. (1994). *Neuron* **13**, 495–503.

De Waard, M., Witcher, D. R., Pragnell, M., Liu, H., and Campbell, K. P. (1995). *J. Biol. Chem.* **270**, 12056–12064.

De Waard, M., Gurnett, C. A., and Campbell, K. P. (1996). *Ion Channels*, Vol. 4 (Toshio Narahashi, ed.), Plenum Press, New York, pp. 41–87.

Gao, T., Puri, T. S., Gerhardstein, B. L., Chien, A. J., Green, R. D., and Hosey, M. M. (1997a). *J. Biol. Chem.* **272**, 19401–19407.

Gao, T., Yatani, A., Dell'Acqua, M., Sako, H., Green, S. A., Dascal,

N., Scott, J. D., and Hosey, M. M. (1997b). *Neuron* **19**, 185–196.
Gurnett, C. A., De Waard, M., and Campbell, K. P. (1996). *Neuron* **16**, 431–440.
Gutierrez, L. M., Zhao, X. L., and Hosey, M. M. (1994). *Biochem. Biophys. Res. Commun.* **202**, 857–865.
Haase, H., Karczewski, P., Beckert, R., and Krause, E. G. (1993). *FEBS Lett.* **335**, 217–222.
Hart, G. W. (1997). *Annu. Rev. Biochem.* **66**, 315–335.
Hell, J. W., Westenbroek, R. E., Breeze, L. J., Wang, K. K., Chavkin, C., and Catterall, W. A. (1996). *Proc. Natl. Acad. Sci. USA* **93**, 3362–3367
Hochstrasser, M. (1996). *Cell* **84**, 813–815.
Hosey, M. M., Chien, A. J., and Puri, T. S. (1996). *Trends Cardiovasc. Med.* **6**, 265–273.
Hullin, R., Singer-Lahat, D., Freichel, M., Biel, M., Dascal, N., Hofmann, F., and Flockerzi, V. (1992). *EMBO J.* **11**, 885–890.
Jay, S. D., Sharp, A. H., Kahl, S. D., Vedvick, T. S., Harpold, M. M., and Campbell, K. P. (1991). *J. Biol. Chem.* **266**, 3287–3293.
Johnson, B. D., Scheuer, T., and Catterall, W. A. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 11492–11496.
Johnson, B. D., Brousal, J. P., Peterson, B. Z., Gallombardo, P. A., Hockerman, G. H., Lai, Y., Scheuer, T., and Catterall, W. A. (1997). *J. Neurosci.* **17**, 1243–1255.
Leung, A. T., Imagawa, T., Block, B., Franzini-Armstrong, C., and Campbell, K. P. (1988). *J. Biol. Chem.* **263**, 994–1001.
Liu, H., De Waard, M., Scott, V. E. S., Gurnett, C. A., Lennon, V. A., and Campbell, K. P. (1996). *J. Biol. Chem.* **271**, 13804–13810.
McDonald, T. F., Pelzer, S., Trautwein, W., and Pelzer, D. J. (1994). *Physiol. Rev.* **74**, 365–507.
Mundina-Weilenmann, C., Chang, C. F., Gutierrez, L. M., and Hosey, M. M. (1991). *J. Biol. Chem.* **266**, 4067–4073.
Mumby, S. (1997). *Curr. Opin. Cell. Biol.* **9**, 148–154.
Olcese, R., Qin, N., Schneider, T., Neely, A., Wei, X., Stefani, E., and Birnbaumer, L. (1994). *Neuron* **13**, 1433–1438.
Perets, T., Blumenstein, Y., Shistik, E., Lotan, I., and Dascal, N. (1996). *FEBS Lett.* **384**, 189–192.
Perez-Reyes, E., Castellano, A., Kim, H. S., Bertrand, P., Baggstrom, E., Lacerda, A. E., Wei, X. Y., and Birnbaumer, L. (1992). *J. Biol. Chem.* **267**, 1792–1797.
Perez-Reyes, E., Yuan, W., Wei, X., and Bers, D. M. (1994). *FEBS Lett.* **342**, 119–123.
Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T. P., and Campbell, K. P. (1994). *Nature* **368**, 67–70.
Puri, T. S., Gerhardstein, B., Zhao, X.-L., Ladner, M. B., and Hosey, M. M. (1997). *Biochemistry* **36**, 9605–9615.
Qin, N., Olcese, R., Zhou, J., Cabello, O. A., Birnbaumer, L., and Stefani E. (1996). *Am. J. Physiol. C* **271**, 1539–1545.
Qin, N., Platano, D., Olcese, R., Costantin, J. L., Stefani, E., and Birnbaumer, L. (1998). *Proc. Natl. Acad. Sci. USA* **95**, 4690–4695.
Scott, V. E., De Waard, M., Liu, H., Gurnett, C. A., Venzke, D. P., Lennon, V. A., and Campbell, K. P. (1996). *J. Biol. Chem.* **271**, 3207–3212.
Tareilus, E., Roux, M., Qin, N., Olcese, R., Zhou, J., Stefani, E., and Birnbaumer, L. (1997). *Proc. Natl. Acad. Sci. USA* **94**, 1703–1708.
Witcher, D. R., De Waard, M., Kahl, S. D., and Campbell, K. P. (1994). *Methods Enzymol.* **238**, 335–348.
Zhao, X. L., Gutierrez, L. M., Chang, C. F., and Hosey, M. M. (1994). *Biochem. Biophys. Res. Commun.* **198**, 166–173.
Zong, X., Schreieck, J., Mehrke, G., Welling, A., Schuster, A., Bosse, E., Flockerzi, V., and Hofmann, F. (1995). *Pflugers Archi.—Eur. J. Physiol.* **430**, 340–347.