Differential Regulated Interactions of Calcium/Calmodulin-Dependent Protein Kinase II with Isoforms of Voltage-Gated Calcium Channel β Subunits†

Chad E. Grueter,[‡,§] Sunday A. Abiria,[||] Yunji Wu,[⊥] Mark E. Anderson,*[,⊥,#] and Roger J. Colbran*[,‡,||,@]

Department of Molecular Physiology and Biophysics, Center for Molecular Neuroscience, Department of Medicine, and Kennedy Center for Research on Human Development, Vanderbilt University Medical Center, Nashville, Tennessee 37232

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ABSTRACT: Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylates the β_{2a} subunit of voltage-gated Ca^{2+} channels at Thr498 to facilitate cardiac L-type Ca^{2+} channels. CaMKII colocalizes with β_{2a} in cardiomyocytes and also binds to a domain in β_{2a} that contains Thr498 and exhibits an amino acid sequence similarity to the CaMKII autoinhibitory domain and to a CaMKII binding domain in the NMDA receptor NR2B subunit (Grueter, C. E. et al. (2006) *Mol. Cell 23*, 641). Here, we explore the selectivity of the actions of CaMKII among Ca^{2+} channel β subunit isoforms. CaMKII phosphorylates the β_{1b}, β_{2a}, β_3, and β_4 isoforms with similar initial rates and final stoichiometries of 6−12 mol of phosphate per mol of protein. However, activated/autophosphorylated CaMKII binds to β_{1b} and β_{2a} with a similar apparent affinity but does not bind to β_3 or β_4. Prephosphorylation of β_{1b} and β_{2a} by CaMKII substantially reduces the binding of autophosphorylated CaMKII. Residues surrounding Thr498 in β_{2a} are highly conserved in β_{1b} but are different in β_3 and β_4. Site-directed mutagenesis of this domain in β_{2a} showed that Thr498 phosphorylation promotes dissociation of CaMKII-β_{2a} complexes in vitro and reduces interactions of CaMKII with β_{2a} in cells. Mutagenesis of Leu493 to Ala substantially reduces CaMKII binding in vitro and in intact cells but does not interfere with β_{2a} phosphorylation at Thr498. In combination, these data show that phosphorylation dynamically regulates the interactions of specific isoforms of the Ca^{2+} channel β subunits with CaMKII.

Voltage-gated Ca^{2+} channel (VGCC)[1] ion selectivity and responsiveness to pharmacological antagonist ligands are defined by the identity of the pore forming α_1 subunit. The biophysical properties are generally modified by differential association of auxiliary β, $\alpha_2\delta$, and γ subunits (*1*−*3*). Four genes encoding β isoforms have been identified (β_{1-4}), each having multiple mRNA splice variants, which differentially modulate the properties and cell surface expression of VGCC complexes (*4*−*6*). In addition, VGCC complexes are further modulated by a variety of post-translational modifications.

The regulatory properties of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) allow integration of signals conveyed by changes in the frequency, duration, and amplitude of intracellular Ca^{2+} transients (*7*). This feature critically depends on Ca^{2+}/calmodulin-dependent autophosphorylation in the autoregulatory domain at Thr286 in CaMKIIα (or Thr287 in other CaMKII isoforms), which confers subsequent autonomous kinase activity (reviewed in refs *8*−*10*). Thus, Ca^{2+} transients induce more prolonged kinase activation depending on specific parameters of Ca^{2+} signals in the local environment. Recent studies demonstrated that localization of CaMKII to specific subcellular microdomains confers distinct downstream phosphorylation events (*11*, *12*). These studies are consistent with the emerging concept that direct interactions of signaling molecules ensures accurate and timely responses to cell stimulation (*13*, *14*). Determining the mechanisms for CaMKII binding to target proteins, such as VGCCs, is thus an important goal for understanding the role of CaMKII in excitable cells.

CaMKII phosphorylates the α and/or β subunits of a variety of VGCCs to modulate Ca^{2+} entry. For example, CaMKII regulates T-type Ca^{2+} channels by binding to and phosphorylating the II−III intracellular loop in the $Ca_V3.2$ α_1 subunit (*15*, *16*). The EF hand motif in the C-terminal domain of the L-type Ca^{2+} channel (LTCC) $Ca_V1.3$ α_1 subunit is also phosphorylated by CaMKII, causing a negative voltage shift in the LTCC current activation (*17*). CaMKII also phosphorylates multiple sites in the LTCC $Ca_V1.2$ α_1 subunit to promote both Ca^{2+}- and voltage-dependent facilitation in heterologous cells (*18*−*20*). In addition to these roles in feedback regulation, CaMKII is involved in cross-

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* Corresponding authors. (M.E.A.) E-mail: mark-e-anderson@uiowa.edu; tel.: (319) 356-2750. (R.J.C.) E-mail: roger.colbran@vanderbilt.edu; tel.: (615) 936-1630; fax: (615) 322-7236.

‡ Department of Molecular Physiology and Biophysics.

§ Current address: Department of Molecular Biology, 6000 Harry Hines Blvd., Room NA8.142, Dallas, TX 75235.

|| Center for Molecular Neuroscience.

⊥ Department of Medicine.

Current address: Department of Internal Medicine, University of Iowa, Carver College of Medicine, Iowa City, IA 52242.

@ Kennedy Center for Research on Human Development.

[1] Abbreviations: CaMKII, calcium/calmodulin-dependent protein kinase II; CaMKAP, CaMKII-associated protein; GK, guanylate kinase-like; GST, glutathione-*S*-transferase; LTCC, L-type VGCC; VGCC, voltage-gated calcium channel.

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talk between Ca^{2+} channels: for example, LTCC activation leads to the depression of R-type Ca^{2+} channels in dendritic spines via a poorly defined CaMKII-dependent mechanism (*21*).

We recently showed that CaMKII-dependent facilitation of cardiac LTCCs is mediated by phosphorylation of the β_{2a} subunit at Thr498 in cardiomyocytes (*22*). Moreover, β_{2a} acts as a CaMKII associated protein (CaMKAP) that directly interacts with CaMKII in vitro and in intact cells. Our findings showed that VGCC regulation may be strongly enhanced or modified by the association of CaMKII with the β_{2a} subunit. In the current study, we demonstrate that CaMKII phosphorylates all of the β subunit isoforms but interacts with β subunits in an isoform specific manner; this CaMKII-β subunit interaction is negatively modulated by phosphorylation of the β subunit.

MATERIALS AND METHODS

Generation of Plasmid Constructs. The open reading frames of the rat VGCC β_{1b}, β_{2a}, β_3, and β_4 subunits (accession numbers X61394, M80545, M88751, and L02315) (generous gifts from Dr. E. Perez-Reyes, University of Virginia) were amplified by PCR and ligated into pGEX-4T1 (Amersham Pharmacia Biotech). The β_{2a} subunit was also subcloned into pFLAG-CMV-2 (Sigma-Aldrich), pIRES (Clontech), and pLenti (Invitrogen). Murine CaMKIIα and rat CaMKIIδ coding sequences were inserted into pcDNA3. The cDNAs encoding β_{2a} were mutated essentially as described in the QuikChange kit (Stratagene). The pcDNA plasmid encoding a constitutively active T287D mutation of myc-CaMKIIδ2 was a generous gift from Dr. E. Olson (UTSW, Dallas).

GST Fusion Protein Expression and Purification. GST fusion proteins were expressed and purified as described in ref *22*. Protein concentrations were determined by Bradford assay (BioRad) using bovine serum albumin as the standard and were confirmed by resolving proteins on SDS-polyacrylamide gels followed by Coomassie-Blue staining.

CaMKII Purification and Autophosphorylation. Recombinant rat CaMKIIδ_2 or mouse CaMKIIα purified from baculovirus infected Sf9 insect cells were autophosphorylated at Thr287 or Thr286, respectively, using ATP or [γ-^{32}P]ATP, essentially as described previously (*23*).

CaMKII Plate Binding Assays. GST fusion proteins in 0.2 mL of plate-binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1% (v/v) Tween-20, 5 mg/mL bovine serum albumin) were incubated for 18−24 h at 4 °C in glutathione-coated wells. After 3 washes with buffer, wells were incubated at 4 °C with the indicated concentrations of ^{32}P-labeled, Thr287 autophosphorylated CaMKIIδ_2 (0.2 mL) for 2 h and then washed (8 times, 0.2 mL of ice-cold buffer). The bound kinase was quantified using a scintillation counter.

To monitor dissociation of preformed CaMKII-β_{2a} complexes, GST-β_{2a} (wild-type or T498A: ≈5 pmol) was immobilized in glutathione-coated multi-well plates (Pierce, Rockford, IL) and then incubated for 2 h at 4 °C with [^{32}P-T^{286}]CaMKIIα (0.25 μM) in binding buffer. Wells were rinsed 8 times in binding buffer, and immobilized complexes were then incubated with 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.25 mg/mL bovine serum albumin, 0.1% Triton X-100, 1 mM dithiothreitol, 10 mM magnesium acetate, with or without 0.5 mM ATP. Soluble/dissociated CaMKII was removed from the wells at the indicated times and quantified by scintillation counting.

CaMKII Gel Overlays. GST fusion proteins (50 pmol) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Approximately equal protein loading was confirmed by staining membranes using Ponceau S. Membranes were blocked and then incubated for 2 h at 4 °C with ^{32}P-labeled, Thr287 autophosphorylated CaMKIIδ_2 (100 nM), essentially as described previously (*24*). After washing, bound CaMKII was quantified using a phosphoimager.

CaMKII Phosphorylation of GST-β Subunits. Purified GST-β subunits (or GST alone as a blank) were incubated at either 30 or 4 °C in 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 0.4 mM [γ-^{32}]ATP (≈500 cpm/pmol) or 0.4 mM ATP containing purified CaMKII. At the indicated times, aliquots were spotted on P81 phosphocellulose papers, washed in water, and counted in a scintillation counter to measure the stoichiometry of phosphorylation. Parallel aliquots were resolved by SDS-polyacrylamide gel analysis, and dried gels were analyzed by autoradiography and/or a phosphoimager followed by densitometry, as described previously (*22*).

Immunoblotting. Samples were resolved on Tris-glycine SDS-polyacrylamide gels and transferred to nitrocellulose membranes using standard methods. Membranes were blocked in 5% (w/v) milk in TTBS (50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.1% (v/v) Tween-20) and then incubated with primary antibodies overnight at 4 °C. After being washed 6 times for >5 min each, membranes were incubated for 1 h at room temperature with horseradish peroxidase conjugated secondary antibody. The washed membranes were developed using enhanced chemiluminescence.

Co-immunoprecipitations from HEK293 Cells. Experiments were performed as described in ref *22*. Briefly, HEK293FT cells were transfected with FLAG-β_{2a} (wild-type, T498A, T498E, or L493A), myc-CaMKIIδ(T287D), and/or the flag vector alone. Cell lysates were immunoprecipitated with FLAG-coated agarose beads (40 μL, Sigma) and then immunoblotted.

Statistics. Data were expressed as mean ± SEM. Paired comparisons were performed using the Student's *t* test. Multiple group comparisons were performed using one-way or two-way ANOVA with Bonferoni post-hoc testing, unless otherwise noted. The null hypothesis was rejected if $p < 0.05$.

RESULTS

CaMKII Efficiently Phosphorylates β_{1-4} Subunits. The highly efficient phosphorylation of the β_{2a} subunit at Thr498 by CaMKII is critical for CaMKII effects on cardiac LTCCs (*22*). To begin to analyze potential effects of CaMKII on the other β isoforms, we assessed the phosphorylation of full-length β subunit isoforms that had been expressed in bacteria as GST fusion proteins. First, incubations were performed at 4 °C to provide an indication of the initial rate of phosphorylation by 10 nM CaMKII (Figure 1A). Under these conditions, CaMKII preferentially phosphorylates

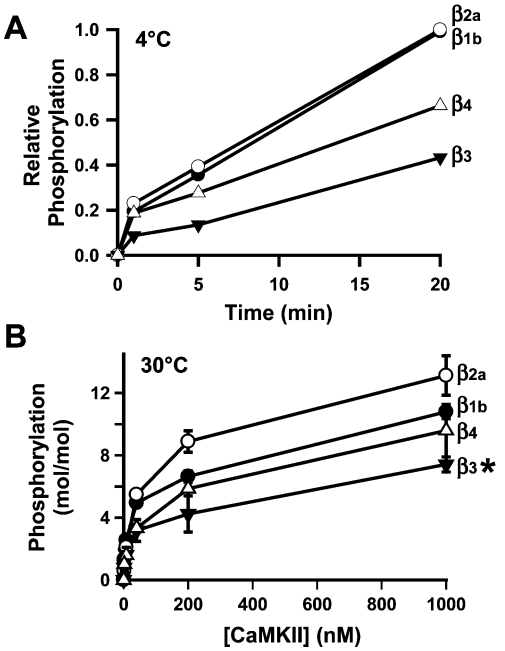

FIGURE 1: CaMKII efficiently phosphorylates all of the β subunit isoforms in vitro. (A) Initial rate of phosphorylation of GST-β isoforms by CaMKIIδ2 at 4 °C. Data (mean of two experiments) are plotted relative to the phosphorylation of GST-β_{2a} after 20 min. GST-β_{2a}, ○; GST-β_{1b}, ●; GST-β_3, ▼; and GST-β_4, △. (B) Stoichiometry of phosphorylation of GST-β isoforms by increasing concentrations of CaMKIIδ2 after 20 min of incubation at 30 °C (mean ± SEM of three experiments). Symbols as in panel A. ∗: Phosphorylation of GST-β_3 by 1000 nM CaMKII was significantly less than that of GST-β_{2a} ($p < 0.05$). Other differences were not statistically significant.

Thr498 in GST-β_{2a} (*22*). All four isoforms were phosphorylated, although β_{1b} and β_{2a} were phosphorylated at a relative rate ≈2.5-fold faster than for β_3 or β_4. We then assessed the extent of phosphorylation by various concentrations of CaMKII using a fixed concentration of the GST-β isoforms (1 μM). We previously identified six CaMKII phosphorylation sites in β_{2a} that had been phosphorylated to a stoichiometry of ≈3 mol/mol (*22*). In the present studies, each isoform was phosphorylated with a similar CaMKII concentration dependence (Figure 1B), with the β_{2a}, β_{1b}, β_3, and β_4 variants attaining stoichiometries of 13.1 ± 1.3, 10.8 ± 0.4, 7.4 ± 0.5, and 9.6 ± 1.7 mol of phosphate/mol of β, respectively. While ANOVA detected significant variability in the final phosphorylation stoichiometries ($p = 0.038$), post-hoc testing revealed a single significant difference between β_3 and β_{2a} phosphorylation stoichiometries ($p < 0.05$). In combination, these data show that these four β subunit isoforms are rapidly phosphorylated by CaMKII to a high stoichiometry in vitro with relatively modest differences in the phosphorylation parameters that were measured.

Selective Interactions of CaMKII with VGCC β Subunits in Vitro. Thr498 of the β_{2a} variant lies within a CaMKII-binding domain that is C-terminal to the SH3 and GK domains (Figure 2A). Alignment of the amino acid sequence of the domain surrounding Thr498 in β_{2a} with other β subunit isoforms revealed variable conservation. A CaMKII consensus phosphorylation motif LXRXXS/T (*25*) is present in both β_{2a} and β_{1b}, and there is additional amino acid sequence similarity outside this motif, but key residues from this motif are missing in β_3 and β_4. On the basis of these alignments, we hypothesized that CaMKII would bind to β_{1b} in a similar

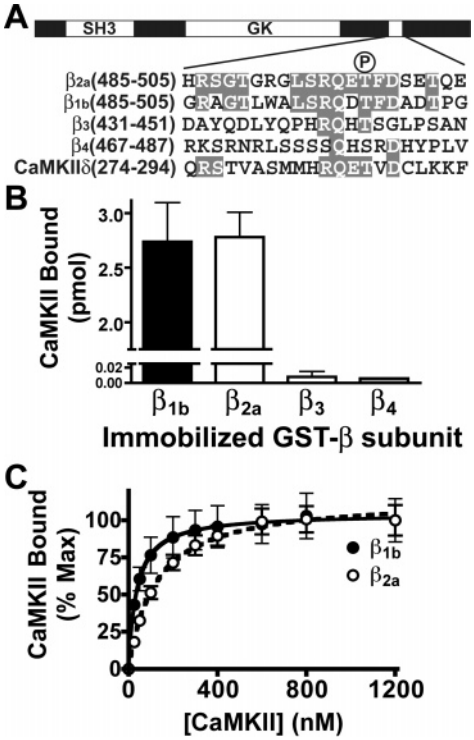

FIGURE 2: CaMKII association with VGCC β subunit isoforms. (A) Schematic domain structure of the β_{2a} subunit showing SH3 and guanylate kinase-like (GK) domains, with the C-terminal CaMKII-binding domain containing the Thr498 phosphorylation site (indicated by the "P"). The amino acid sequence of the CaMKII binding domain in β_{2a} is aligned with similar sequences from other β isoforms and the CaMKIIδ autoregulatory domain (surrounding Thr287): identical residues are shown in gray boxes. (B) GST-β subunit isoforms were immobilized in glutathione-coated multi-well plates (100 pmol/well) and incubated with ^{32}P-labeled Thr287 autophosphorylated CaMKIIδ2 (50 nM subunit). (C) Concentration-dependent binding of Thr287 autophosphorylated CaMKIIδ2 to GST-β_{2a} (○) and GST-β_{1b} (●) in glutathione-coated multi-well plates. Both panels B and C plot binding as mean ± SEM from four observations (β_{2a} and β_{1b}) or the mean of two observations (β_3 and β_4).

manner to its interaction with β_{2a} but not to β_3 or β_4. To test this hypothesis, we immobilized GST-β fusion proteins in glutathione-coated 96-well plates and then incubated them with various concentrations of purified ^{32}P-autophosphorylated CaMKII. In an initial experiment, the binding of CaMKII to GST-β_{1b} was indistinguishable from the binding to GST-β_{2a}, but binding to GST-β_3 and GST-β_4 was <1% of the binding to GST-β_{2a} (Figure 2B). Binding of Thr286-autophosphorylated CaMKII to both GST-β_{1b} and GST-β_{2a} was concentration dependent and saturable (Figure 2C). GST-β_{1b} exhibited a significantly higher apparent affinity for CaMKII than did GST-β_{2a} (apparent K_d values of 35 ± 12 and 120 ± 21 nM CaMKII subunit, respectively, $n = 4$, $p < 0.01$). GST-β_3 and GST-β_4 failed to bind significant amounts of the kinase even at a concentration of 1200 nM CaMKII subunit (data not shown). Autophosphorylated CaMKII also binds to GST-β_{1b} and GST-β_{2a} but not to GST-β_3 and GST-β_4 in glutathione agarose co-sedimentation assays, but there was no significant binding of non-phosphorylated CaMKII to any GST-β subunit isoforms (Supporting Information Figure 1). Thus, CaMKII does not appear to bind significantly to the β_3 and β_4 isoforms but interacts with the β_{1b} isoform with an ≈3-fold higher affinity

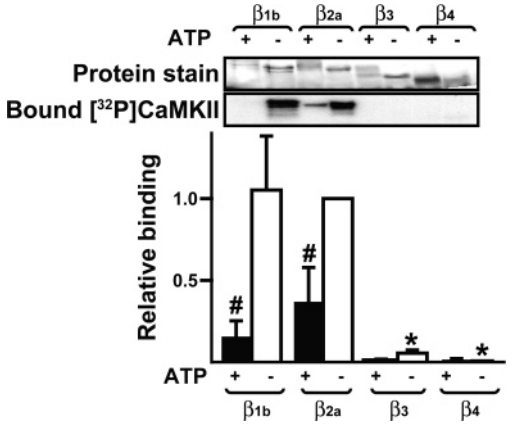

FIGURE 3: Prephosphorylation of GST-β_{2a} and GST-β_{1b} inhibits CaMKII binding. GST-β isoforms were preincubated with activated CaMKIIδ2 in the presence or absence of ATP, as indicated. Reactions were resolved by SDS-PAGE and transferred to a nitrocellulose membrane for overlay with ^{32}P-labeled Thr287 autophosphorylated CaMKIIδ2. The top panel shows a protein stain of the membrane prior to overlay and a representative autoradiograph to detect bound CaMKII. Binding was quantified using a phosphoimager and normalized to the binding detected using non-phosphorylated GST-β_{2a}: the mean ± SEM from three experiments is plotted. Data were analyzed by 2-way ANOVA: ∗: $p < 0.001$ vs binding to non-phosphorylated GST-β_{2a} and #: $p < 0.05$ vs binding to the corresponding non-phosphorylated protein.

than with β_{2a} following activation by Thr286/7 autophosphorylation.

Interaction with CaMKII Is Modulated by Phosphorylation in Vitro. Thr498 and several additional serine and threonine residues lie within the CaMKII binding domain of β_{2a}, suggesting that the interaction with CaMKII may be modulated by phosphorylation. Therefore, GST-β isoforms were preincubated with activated CaMKII in the presence or absence of ATP and then separated from reaction components by SDS-PAGE. The electrophoretic mobility of each GST-β isoform was reduced following preincubation with ATP and CaMKII (Figure 3: protein stain), consistent with the relatively high phosphorylation stoichiometry under these conditions (cf., Figure 1B). An overlay assay was then used to assess the binding of ^{32}P-autophosphorylated CaMKII. Non-phosphorylated GST-β_{1b} and GST-β_{2a} proteins bound substantial, but comparable, amounts of CaMKII (Figure 3). Pre-phosphorylation significantly reduced CaMKII binding to both proteins by 70−80%. No significant interactions were seen between CaMKII and either GST-β_3 or GST-β_4, whether or not these protein were pre-phosphorylated. In combination, these data suggest that phosphorylation of the β subunit by CaMKII modulates the binding of CaMKII to the β_{1b} and β_{2a} isoforms.

Mechanism of CaMKII Binding to β_{2a}. To dissect the mechanism for CaMKII binding, we assessed the effect of mutating residues within the CaMKII binding domain of β_{2a} on the interaction with CaMKII. Mutation of Thr498 to Ala or Glu prevented or mimicked phosphorylation at this site, respectively. In addition, residues homologous to Leu493 at the p-5 position relative to Thr498 in β_{2a} and β_{1b} are conserved in other high affinity CaMKII phosphorylation sites that form stable complexes with the CaMKII catalytic domain prior to phosphorylation (e.g., Ser1303 in NR2B and Thr286/7 in CaMKIIα/δ). However, hydrophobic residues at the p-5 position are not conserved in β_3 or β_4 and are not generally considered to be part of the minimal consensus phosphorylation site (*25*). Therefore, we also mutated Leu493 to Ala. Binding of ^{32}P-autophosphorylated CaMKII to GST-β_{2a} in glutathione-coated multi-well plates was unaffected by the T498A mutation, whereas the T498E and L493A mutations significantly reduced CaMKII binding by >90% (Table 1). These data demonstrate that the region surrounding Thr498 is critical for stable binding of Thr286-autophosphorylated CaMKII to the full-length β_{2a} isoform.

Table 1: Disruption of CaMKII Binding to GST-β_{2a} by Site-Directed Mutagenesis[a]

GST-β_{2a} protein	CaMKII bound (pmol of subunit)
wild-type	7.9 ± 0.3
T498A	6.2 ± 0.8
T498E	0.51 ± 0.16
L493A	0.14 ± 0.03

[a] Purified GST-β_{2a} proteins (wild-type or with the indicated point mutations) or GST alone were immobilized in a glutathione-coated 96-well plate (100 pmol/well) and then incubated with ^{32}P-labeled Thr287-autophosphorylated CaMKIIδ_2 (100 nM subunit). After washing, bound CaMKII was quantified by scintillation counting. The data indicate the mean ± SEM ($n = 3$ experiments).

Disruption of CaMKII Binding Does Not Affect Phosphorylation of β_{2a} in Vitro. We then explored the effects of the Thr498 and Leu493 mutations on CaMKII phosphorylation of the β_{2a} subunit. The T498A mutation substantially reduced the rate of β_{2a} phosphorylation at 4 °C, as shown previously, and T498E mutation had a very similar effect. Interestingly, the rate of L493A-β_{2a} phosphorylation by CaMKII was indistinguishable from the rate of phosphorylation of the wild-type protein (Figure 4A), even though the L493A mutation interfered with CaMKII binding. When incubations were conducted at 30 °C, CaMKII (10 nM) phosphorylated wild-type β_{2a} to a stoichiometry of 2.2 mol/mol, consistent with stoichiometries reported in Figure 1B using 10 nM CaMKII, but T498A mutation reduced phosphorylation to 0.3 mol/mol (Figure 4B). In contrast, T498E and L493A mutations had no significant effect on the stoichiometry of CaMKII phosphorylation, although there was a trend for reduced phosphorylation of T498E-β_{2a}. These data show that two mutations that severely compromise stable binding of activated CaMKII to β_{2a} have no significant effect on the overall stoichiometry of CaMKII phosphorylation in vitro.

As noted previously, Leu493 is part of a conserved sequence surrounding high-affinity phosphorylation sites in multiple CaMKII-binding proteins. To determine as to whether the L493A mutation altered the phosphorylation site specificity of CaMKII, we exploited similarities in protein sequences surrounding Thr498 in β_{2a} and Thr287 in CaMKIIδ (Figure 2A). Commercially available phospho-Thr287 antibodies detected the β_{2a} subunit following CaMKII phosphorylation but not nonphosphorylated β_{2a} (Figure 4B and data not shown). Phospho-Thr286/7 antibodies only weakly detected phosphorylated β_{1b} and failed to detect β_3 or β_4 before or after CaMKII phosphorylation (data not shown). Analysis of mutated β_{2a} proteins revealed that T498A-β_{2a} and T498E-β_{2a} could not be detected (Figure 4B, middle blot), demonstrating that the antibody specifically recognized phospho-Thr498 in β_{2a}, in addition to the Thr286/287 autophosphorylation site in CaMKII. The lack of antibody cross-reactivity with T498E-β_{2a} is not surprising given

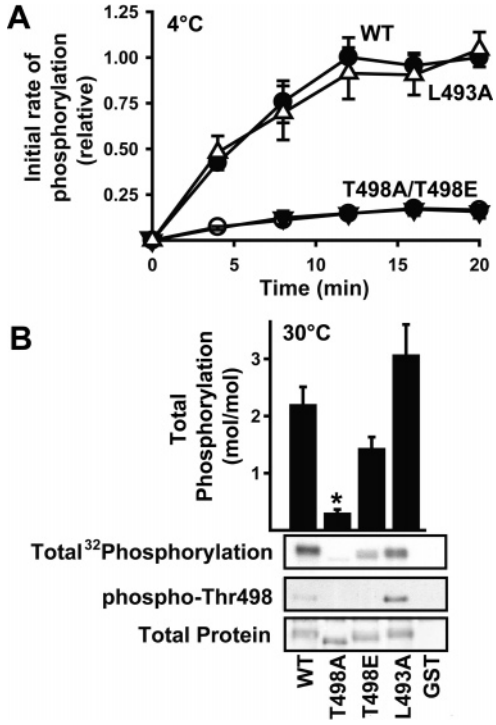

FIGURE 4: Phosphorylation of GST-β_{2a} by CaMKII is independent of stable binding. (A) Initial rate for phosphorylation of GST-β_{2a} (wild type, ●; T498A, ▼; T498E, ○; and L493A, △) by CaMKII (10 nM) at 4 °C. Data are the mean ± SEM of three to four observations normalized to the phosphorylation of the wild-type protein after 20 min. (B) Wild-type or mutated GST-β_{2a} proteins or GST alone were incubated for 20 min at 30 °C with activated CaMKII (10 nM) and [γ-^{32}P]ATP. Aliquots of the reactions were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were stained for total protein (bottom) and then immunoblotted using a phospho-specific antibody that was raised to phospho-Thr286 in CaMKIIα but also detects phospho-Thr498 in β_{2a} (middle). Total phosphorylation of β_{2a} was detected by autoradiography (top gel). The stoichiometry of total phosphorylation (bar graph) was estimated after spotting parallel aliquots on phosphocellulose papers (see Materials and Methods). Data are plotted as mean ± SEM ($n = 4$ experiments). ∗: $p < 0.01$ vs wild-type.

differences in size and charge density between glutamate and phospho-threonine. Taken together, these findings validate the use of this antibody to report the phosphorylation status of Thr498 in the CaMKII binding domain of β_{2a}. Notably, Thr498 was phosphorylated to a similar extent in L493A-β_{2a} and wild-type β_{2a} (Figure 4B, inset). Thus, the L493A mutation does not appear to significantly alter the ability of CaMKII to phosphorylate Thr498 in vitro.

Phosphorylation at Thr498 Disrupts CaMKII Binding. To explore the mechanism by which phosphorylation of β_{2a} by CaMKII interferes with subsequent binding of activated CaMKII, we pre-phosphorylated wild-type and mutated β_{2a} proteins and analyzed CaMKII binding using overlay assays. As seen using the glutathione plate-binding assay, T498A-β_{2a} bound comparable amounts of activated CaMKII to wild-type β_{2a}. However, pre-phosphorylation of T498A-β_{2a} had no significant effect on binding of activated CaMKII, whereas pre-phosphorylation of wild-type β_{2a} significantly reduced binding by ≈80% in these assays. The T498E mutation reduced CaMKII binding by ≈80%, and CaMKII phosphorylation had no significant additional effect on binding to T498E-β_{2a}. Interestingly, the L493A mutation

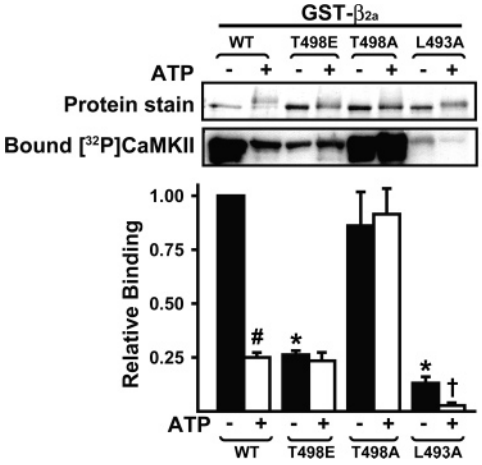

FIGURE 5: Prephosphorylation of β_{2a} at Thr498 inhibits the association of CaMKII. (A) Wild-type and mutated GST-β_{2a} proteins were preincubated with activated CaMKIIδ2 in the presence or absence of ATP. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes to detect total protein loaded (top) and to probe with [^{32}P]CaMKIIδ2 by overlay assay and autoradiography (middle). Binding was quantified using a phosphoimager and normalized to binding to non-phosphorylated wild-type protein: the mean ± SEM from three experiments is plotted. #: $p < 0.001$ vs corresponding non-phosphorylated protein and ∗: $p < 0.001$ vs non-phosphorylated GST-β_{2a} wild-type. Further post-analyses using *t* tests showed that prephosphorylation significantly reduced CaMKII binding to the L493A mutant (†: $p < 0.05$).

significantly reduced binding by ≈90% in these assays, and pre-phosphorylation by CaMKII resulted in an additional significant decrease in binding. Taken together, these data suggest that pre-phosphorylation of β_{2a} at Thr498 substantially reduces the association of CaMKII.

We also investigated the role of Thr498 phosphorylation in the context of preformed CaMKII-β_{2a} complexes. Complexes of autonomously active Thr286 autophosphorylated CaMKII bound to GST-β_{2a} (wild-type) were isolated in glutathione-coated multi-well plates and then incubated with or without the addition of ATP. The addition of ATP to these complexes induced phosphorylation at Thr498 and several other sites, as reflected by immunoblotting with phospho-Thr286 CaMKII antibodies and by a substantial reduction in the electrophoretic mobility of GST-β_{2a} (data not shown). In the absence of ATP, CaMKII complexes with wild-type β_{2a} were remarkably stable (<5% dissociation over a 2 h incubation), but the addition of ATP induced dissociation of ≈50% of bound CaMKII. The T498A mutation of β_{2a} had little effect on the stability of complexes with CaMKII in the absence of ATP but substantially reduced the rate and extent of dissociation following addition of ATP as compared to wild-type β_{2a} (Figure 6). However, ATP still enhanced the dissociation of CaMKII from preformed complexes with T498A-β_{2a} (≈20% in 2 h), suggesting that phosphorylation at other residues in β_{2a} and/or CaMKII may have a modest effect on the stability of these complexes, at least in vitro. In combination, these data suggest that phosphorylation of β_{2a} at Thr498 enhances the dissociation of CaMKII from preformed complexes with β_{2a} in vitro.

CaMKII Interaction with β_{2a} Is Regulated in Cells. Initially, we explored as to whether Thr498 in β_{2a} is phosphorylated in intact cells. Lysates of HEK293 cells expressing FLAG-β_{2a} (wild-type or mutated) with a constitutively active CaMKIIδ_2 mutant were immunoprecipitated

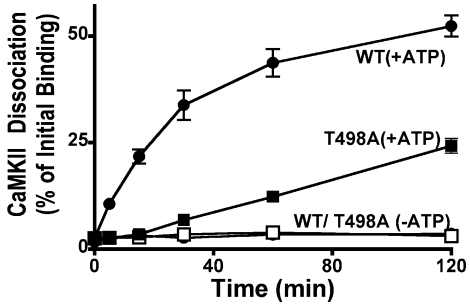

FIGURE 6: Phosphorylation of β_{2a} at Thr498 enhances dissociation of the CaMKII-β_{2a} complex. Complexes of GST-β_{2a} (wild-type or T498A) and [^{32}P-T^{286}]CaMKIIα in glutathione-coated multi-well plates were incubated with or without ATP in a dissociation buffer (see Materials and Methods). At the indicated times, dissociated [^{32}P-T^{286}]CaMKIIα was removed from the wells and quantified by scintillation counting. Data points represent mean ± SEM ($n = 3$) ($n = 2$ for β_{2a} T498A in the absence of ATP): error bars lie within symbols of some data points.

using FLAG antibodies. Blotting the immune complexes with FLAG antibodies revealed a relatively consistent expression and immunoprecipitation of the β_{2a} proteins. The phospho-Thr286 CaMKIIα antibodies detected the β_{2a} wild-type and L493A mutant but not the T498A- or T498E-β_{2a} proteins (Figure 7A). These data show that Thr498 can be phosphorylated to a similar extent in wild-type β_{2a} and L493A-β_{2a} in intact cells.

To investigate the regulation of CaMKII interaction with β_{2a} in intact cells by modification of Thr498 and the surrounding domain, lysates of HEK293 cells expressing constitutively active myc-tagged CaMKIIδ_2 without or with FLAG-tagged β_{2a} subunits (wild-type or mutated) were immunoprecipitated using FLAG antibodies. CaMKII was readily detected in immune complexes containing FLAG-tagged wild-type β_{2a} or T498A-β_{2a} but could not be detected in immune complexes formed by T498E-β_{2a} of L493A-β_{2a} (Figure 7B). These findings demonstrate that the domain surrounding Thr498 is critical for the association of CaMKII with β_{2a} in cells and suggest that phosphorylation at Thr498 diminishes CaMKII binding to β_{2a} in cells.

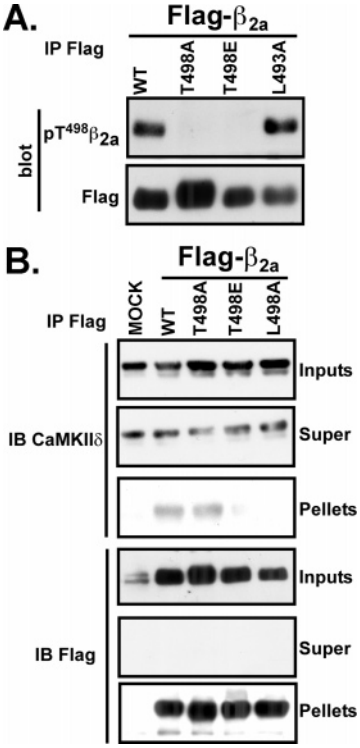

FIGURE 7: CaMKII interaction with β_{2a} is regulated by Thr498 phosphorylation in situ. Myc-tagged T287D-CaMKIIδ2 was co-expressed with or without FLAG-tagged wild-type or mutated β_{2a} proteins in HEK293 cells. Aliquots of the cell lysates (inputs), FLAG immune supernatants (super), and FLAG immune complexes (pellets) were immunoblotted. (A) Probing FLAG immune complexes using antibodies raised to phospho-Thr286 in CaMKIIα showed that wild-type and L493A β_{2a} are partially phosphorylated at Thr498 in intact cells. (B) Probing FLAG immune complexes using CaMKIIδ antibodies revealed that CaMKII was associated with wild-type and T498A β_{2a} but could not be detected in the T498E and L493A β_{2a} immune complexes. The data are representative of >4 experiments.

DISCUSSION

A diverse family of VGCCs regulates Ca^{2+} entry into excitable and non-excitable cells. The α_1 subunits confer core biophysical and pharmacological behavior of each type of VGCC. However, a cytosolic loop between the first and the second major transmembrane domains in the α_1 subunit is generally thought to constitutively interact with a β subunit. Alternative splicing of mRNAs from four mammalian genes generates >20 distinct β subunit proteins that have divergent roles in modulating the trafficking and biophysical properties of VGCCs (*6*). The β subunits share highly conserved SH3 and GK domains that form a compact structure, with a hydrophobic groove in the GK domain that interacts with the α_1 subunit (*26*). However, some β subunits lack substantial parts of the SH3 and GK domains, yet still modulate LTCCs (*27*). These observations support recent findings, showing that additional SH3-GK independent modulatory interactions between the α and the β subunits are important for regulating VGCC activity (*28*−*31*). The variable domains presumably account for the unique effects of β subunit variants on the properties of α_1 subunits.

Most studies have focused on the roles of protein interactions and post-translational modifications of the α_1 subunit in modulating VGCCs. However, the importance of β subunits in regulating VGCCs has received increasing attention. Initial studies suggested that PKA phosphorylation of the β_2 subunit plays a role in facilitating LTCCs (*32*), although the importance of this modification in native cells has recently been questioned (*33*). In addition, β subunits have been shown to serve as scaffolding proteins that bind REM GTPases to inhibit VGCCs (*34*, *35*) and AHNAKs to link VGCCs to the actin cytoskeleton (*36*). Our recent work showed that CaMKII binds β_{2a} subunits and colocalizes with β_{2a} in adult cardiomyocytes. Moreover, phosphorylation of β_{2a} at Thr498 is required for Ca^{2+}- and CaMKII-dependent facilitation of LTCCs in cardiomyocytes (*22*). While β subunit variants have unique direct effects on the biophysical properties and trafficking of LTCCs, the β isoform selectivity of regulation by CaMKII and other modulators is poorly understood.

The β subunit variants tested here associate with multiple VGCC α_1 subunits (reviewed in refs *2* and *6*). Thus, our current results showing that activated/Thr286 autophosphorylated CaMKII forms stable complexes with β_{1b} and β_{2a}, but not with β_3 or β_4, lead us to hypothesize that the association of CaMKII with VGCC complexes will depend on the identity of the associated β subunit. The β_{1b} and β_{2a}

subunits contain an LXRXXS/T motif similar to sequences surrounding phosphorylation sites in NR2B and the CaMKII autoinhibitory domain that also form stable complexes with the CaMKII catalytic domain. Mutation of Leu493 to Ala within this motif reduced CaMKII binding to β_{2a} by >90% but surprisingly had little effect on the initial rate of phosphorylation at Thr498 or on the overall phosphorylation stoichiometry at multiple sites. The lack of conservation of this motif accounts for the failure to detect binding to β_3 or β_4.

Despite the apparent binding selectivity, CaMKII phosphorylates all four β subunit variants tested here with comparable relative rates and overall extent. The high maximal phosphorylation stoichiometries suggest that CaMKII efficiently phosphorylates several sites in each β isoform in vitro. Indeed, we previously identified six CaMKII phosphorylation sites in β_{2a} (*22*), and it will be important to identify phosphorylation sites in other β isoforms and to determine their impact on the properties of VGCCs. Data presented here show that phosphorylation at Thr498 in β_{2a} negatively regulates CaMKII binding both in vitro and in situ. Presumably, the conserved LXRXXS/T motif in β_{1b} is responsible for the regulated binding of CaMKII to this variant. Thus, interactions of CaMKII with β_2 and/or β_1 variants are regulated by phosphorylation, likely playing an important role in modulating CaMKII targeting to LTCCs and/or other VGCCs.

Interestingly, β subunits are not generally thought to be important in the regulation of T-type VGCCs. However, recent studies show that CaMKII directly interacts with and phosphorylates the II−III linker of the $Ca_V3.2$ α_1 subunit, shifting the current−voltage activation curve (*15*, *16*). In addition, recent data suggest that CaMKII may also interact with multiple cytoplasmic domains in $Ca_V1.2$ (*19*). Thus, association of CaMKII with VGCC α_1 subunits may be an additional important feature allowing localized Ca^{2+} concentrations to feedback and regulate Ca^{2+} influx.

Emerging data over the last couple of years suggest that mechanisms underlying CaMKII modulation of LTCCs are complex. Phosphorylation of multiple sites in the $Ca_V1.2$ α_1 subunit by CaMKII appears to play a distinct role in voltage- and calcium-dependent modulation in heterologous cells (*18*, *20*). In addition, interactions of CaMKII with multiple intracellular domains of the $Ca_V1.2$ α_1 subunit have been implicated in LTCC facilitation (*19*). In contrast, phosphorylation at Thr498 in β_{2a} is required for CaMKII to increase channel open probability at the single channel level and to facilitate whole cell Ca^{2+} currents in adult cardiomyocytes (*22*). The present observations that phosphorylation at Thr498 appears to be relatively independent of the stable binding of CaMKII to β_{2a} (Figure 4), and also inhibits CaMKII binding (Figures 3, 5, and 6), provides insight that may reconcile these seemingly disparate observations. Interestingly, phosphorylation of β_{2a} in preformed complexes appears to promote the dissociation of CaMKII (Figure 6). CaMKII dissociation might be required to allow protein phosphatases to act on the phospho-Thr498 site to reset channels to their basal state. Presumably, such a mechanism would be most relevant if Thr498 phosphorylation directly modulates LTCC properties, as suggested by the importance of Thr498 in CaMKII-mediated increases in LTCC open probability and LTCC current facilitation in adult cardiomyocytes (*22*). Second, CaMKII may serve a structural role to dynamically assemble complexes containing additional, as yet unidentified, proteins associated with LTCCs. Thus, Thr498 phosphorylation in β_{2a} may promote reorganization of these protein complexes to enable facilitation. Structural roles for CaMKII have been postulated in neuronal post-synaptic densities (*23*). Previous studies showed that disruption of the actin- or tubulin-based cytoskeleton essentially abrogated CaMKII-dependent facilitation of cardiac LTCCs without affecting PKA-dependent facilitation, suggesting an important role for higher orders of molecular organization close to LTCCs in mediating the effects of CaMKII (*37*). A final possibility is that dissociation from β_{2a} induced by Thr498 phosphorylation is important in allowing activated CaMKII to phosphorylate nearby regulatory sites in the α_{1c} or β subunits and/or other associated proteins. In other words, stable binding of activated CaMKII to β_{2a} may limit access of phosphorylation sites in other proteins to the CaMKII active site. Consistent with this hypothesis, dissociation of CaMKII from β_{2a} is substantially reduced by T498A mutation, perhaps explaining as to why CaMKII phosphorylates this mutant to a lower final stoichiometry than T498E-β_2 and L493A-β_2, which have much weaker interactions with CaMKII (Figure 4B). Further studies will be needed to clarify the mechanism of CaMKII actions at VGCC complexes and, perhaps, to identify new CaMKII targets.

In summary, findings reported here and in other recent papers suggest that feedback regulation of Ca^{2+} influx via VGCCs is precisely controlled in specific subcellular microdomains by multiple mechanisms that allow CaMKII and other Ca^{2+}-dependent signaling proteins to associate with channel subunits. The precise nature of the feedback regulation by CaMKII seems likely to depend on the identity of the β subunit associated with the complex. The regulated interaction of activated CaMKII with β_1 and β_2 variants seems likely to be important, although phosphorylation of β_3 and β_4 may also play a role in some cases. Our findings are in line with recent studies, suggesting that subcellular targeting of CaMKII via its interactions with CaMKAPs modulates the specificity of its downstream actions (*11*, *12*). These complex biochemical mechanisms for feedback regulation of Ca^{2+} influx via VGCCs presumably provide great flexibility for modulating a variety of downstream signaling events such as cardiac excitation−contraction and excitation−transcription coupling and neuronal synaptic plasticity. Moreover, alterations in the association of β subunits with VGCCs might disrupt feedback regulation and downstream signaling in heart failure and other diseases (*38*).

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SUPPORTING INFORMATION AVAILABLE

CaMKII binding to GST-β_{1b} and GST-β_{2a} in a glutathione agarose co-sedimentation assay is dependent on prior autophosphorylation of CaMKII (Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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