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CaMKII associates with $Ca_V1.2$ L-type calcium channels via selected β subunits to enhance regulatory phosphorylation

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Abstract

Calcium/calmodulin-dependent kinase II (CaMKII) facilitates L-type Calcium Channel (LTCC) activity physiologically, but may exacerbate LTCC-dependent pathophysiology. We previously showed that CaMKII forms stable complexes with voltage-gated calcium channel β_{1b} or β_{2a} subunits, but not with the β_3 or β_4 subunits (Grueter et al 2008, Biochemistry, **47**:1760–1767). CaMKII-dependent facilitation of $Ca_V1.2$ LTCCs requires Thr498 phosphorylation in the β_{2a} subunit (Grueter et al, 2006, Mol Cell, **23**:641–650), but the relationship of this modulation to CaMKII interactions with LTCC subunits is unknown. Here we show that CaMKII co-immunoprecipitates with forebrain LTCCs that contain $Ca_V1.2\alpha_1$ and β_1 or β_2 subunits, but is not detected in LTCC complexes containing β_4 subunits.4 CaMKIIα can be specifically tethered to the I/II linker of $Ca_V1.2\alpha_1$ subunits *in vitro* by the β_{1b} or β_{2a} subunits. Efficient targeting of CaMKIIα to the full-length $Ca_V1.2\alpha_1$ subunit in transfected HEK293 cells requires CaMKII binding to the β_{2a} subunit. Moreover, disruption of CaMKII binding substantially reduced phosphorylation of β_{2a} at Thr498 within the LTCC complex, without altering overall phosphorylation of $Ca_V1.2\alpha_1$ and β subunits. These findings demonstrate a biochemical mechanism underlying LTCC facilitation by CaMKII.

Keywords

calcium channel; CaMKII; facilitation; phosphorylation; protein-protein interaction

INTRODUCTION

Voltage-activated L-type calcium channels (LTCCs) generate Ca^{2+} signals for multiple physiological processes, including muscle contraction, neurotransmission, neuronal plasticity, dendritic arborization, gene expression and secretion (Catterall 2000). Electrophysiological and pharmacological properties of LTCCs are determined by their pore-forming α_1 subunits ($Ca_V1.1$ through $Ca_V1.4$), but auxiliary subunits (β_{1-4}, α_2-δ and γ) modulate their trafficking, surface expression and biophysical properties (reviewed in (Birnbaumer *et al.* 1998, Catterall 2000, Dolphin 2003)). Most splice variants of the β subunits contain SH3 and Guanylate Kinase (GK)-like domains that are typical of membrane-associated guanylate kinase (MAGUK) family scaffolding proteins. The GK-like domains are responsible for primary interaction of

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β subunits with the Alpha Interacting Domain (AID) located in the cytosolic I/II linker region of α_1 subunits (Van Petegem *et al.* 2004). Thus, LTCC β subunits make ideal molecular scaffolds for anchoring cytosolic regulatory proteins to fine-tune Ca^{2+} signals near LTCCs. These interactions contribute to the plethora of molecular interactions that have been implicated in regulating LTCC activity (reviewed in (Hidalgo & Neely 2007, Calin-Jageman & Lee 2008, Dai *et al.* 2009).

The multifunctional calcium/calmodulin (Ca^{2+}/CaM)-dependent protein kinase II (CaMKII) is a key downstream effector of LTCC signals. In myocytes, CaMKII is required for normal coupling of excitation to contraction (reviewed in (Couchonnal & Anderson 2008, Bers 2008)) and transcription (Wu *et al.* 2006, Zhang *et al.* 2007, Bossuyt *et al.* 2008). Furthermore, CaMKII was recently shown to regulate excitation-transcription coupling in neurons (Wheeler *et al.* 2008). All isoforms of CaMKII are activated by Ca^{2+}/CaM binding and undergo rapid autophosphorylation at Thr286/7 to generate a Ca^{2+}-independent form with enhanced affinity for several CaMKII-associated proteins that may target CaMKII to specific subcellular compartments (Griffith *et al.* 2003, Colbran 2004, Merrill *et al.* 2005). In addition to these downstream actions, CaMKII doubles as a feedback regulator of LTCCs (Anderson *et al.* 1994, Yuan & Bers 1994, Xiao *et al.* 1994). CaMKII participates in several forms of $Ca_V1.2$ LTCC facilitation, including voltage-dependent facilitation induced by a single, strong depolarization, Ca^{2+}-dependent facilitation induced during mild repeated depolarizations, and an increase in channel open probability (Anderson et al. 1994, Xiao et al. 1994, Lee *et al.* 2006, Dzhura *et al.* 2000). Hormones and neurotransmitters can also facilitate both $Ca_V1.2$ and $Ca_V1.3$ LTCCs by CaMKII-dependent mechanisms (O-Uchi *et al.* 2005, Gao *et al.* 2006).

LTCCs are localized in specific subcellular compartments that also contain CaMKII, such as at the t-tubules (z lines) of cardiomyocytes or in neuronal dendritic spines (Gao et al. 2006, Grueter *et al.* 2006, Lee *et al.* 2009, Yasuda *et al.* 2003, Wu *et al.* 1999, O-Uchi et al. 2005). CaMKII-dependent LTCC facilitation is thought to require local Ca^{2+}-dependent activation of CaMKII at the channel because facilitation can be blocked by a "fast" Ca^{2+} chelator (BAPTA), but not a slow chelator (EGTA) (Xiao et al. 1994). Indeed, CaMKII associated with the cytosolic face of membrane patches excised from cardiomyocytes can regulate LTCC open probability. In addition, cytoskeletal disruption interferes with $Ca_V1.2$ LTCCs regulation by CaMKII in cardiomyocytes (Dzhura *et al.* 2002). However, the precise molecular basis for CaMKII targeting to and regulation of LTCCs remains unresolved.

Studies in heterologous cells suggest that CaMKII may directly bind to and/or phosphorylate the $Ca_V1.2\alpha_1$ subunit to support multiple forms of LTCC facilitation (Hudmon *et al.* 2005, Lee et al. 2006, Erxleben *et al.* 2006). However, β subunits have been shown to play an important role in LTCC facilitation (Kamp *et al.* 2000, Bourinet *et al.* 1994, Zhang *et al.* 2005). We recently showed that phosphorylation of Thr498 in the β_{2a} subunit is essential for facilitation of native LTCCs in cardiomyocytes (Grueter et al. 2006). We also showed that CaMKII can form stable complexes with β_{1b} and β_{2a} subunits but not with β_3 or β_4 subunits (Grueter *et al.* 2008). Thus, β_1 and β_2 subunits may be key loci for CaMKII-dependent regulation of LTCCs.

Here, we explored the role of β subunits in targeting CaMKII to LTCC α_1 subunits. We show that CaMKII selectively associates with forebrain LTCCs containing β_1 or β_2 subunits, but not the β_4 subunit. Studies in heterologous cells demonstrate that CaMKII interaction with the β subunit is critical for assembly with the LTCC complex and for phosphorylation of a key regulatory site in β_{2a} (Thr498) within these complexes.

MATERIALS AND METHODS

Generation of plasmid constructs

A pLenti6-V5-D-Topo (Invitrogen, Carlsbad, CA) construct containing the rabbit cardiac $Ca_V1.2\alpha_1$ open reading frame (Accession Number X15539) with a surface HA-epitope between amino acids F709 and D710 of domain II S5-H5 extracellular loop (hereafter called HA-$Ca_v1.2\alpha_1$) was a generous gift of Dr. William Thiel (Thiel *et al.* 2008). The I/II linker was amplified from the parent construct and inserted into a pGEX 4T-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden). The pGEX 4T-1 encoding the $Ca_v1.2$ AID was a generous gift of Dr. Rong Zhang. The open reading frames of the rat β_{1b}, β_{2a}, β_3 and β_4 (Accession Numbers X61394, M80545.1, M88751 and L02315 respectively) were generous gifts from Dr. Timothy Kamp and Dr. Ed Perez-Reyes. The pGEX4T-1 and pLenti6-V5-D-Topo vectors for expression of β subunits were described previously (Grueter et al. 2006, Grueter et al. 2008). The β subunit cDNAs were also amplified by PCR and ligated into pGEX4T-1, pRSET A (Invitrogen), pFLAG-CMV2 (Sigma-Aldrich) and pHA-CMV5 (Clontech) for expression of affinity-tagged β subunits in bacteria or HEK293 cells. The pEGFP (Clonetech, Mount View, CA) construct encoding murine CaMKII with an N-terminal EGFP tag was made by Dr. Stefan Strack. Site-directed mutagenesis was performed essentially as described in the QuikChange kit (Stratagene, La Jolla, CA).

Preparation of forebrain samples

The following procedure for preparation of Triton X-100 soluble fractions from rat or mouse brains was modified from Davare et al (Davare *et al.* 1999). All procedures were performed at 4°C. Forebrains from 7- to 8-week old Sprague-Dawley rats (Pel-Freeze Biologicals, Rogers, Arkansas) or from 12- to 14-week old C57B6J (Jackson Labs, West Grove, Pennsylvania) were pulverized under liquid nitrogen. The frozen powder was homogenized in Teflon-glass Potter-Elvehjem tissue grinders (Kontes, Vineland, NJ) in 5 to 10 ml of Solubilization Buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100 (v/v), 20 mM EDTA, 10 mM EGTA, 100 μM PMSF, 500 μM benzamidine, 0.25 μM microcystin LR, 20 mM sodium β-glycerophosphate, 50 mM sodium fluoride, and protease inhibitor cocktail (Sigma, Catalog # P2714)). Homogenates were sonicated for one minute and centrifuged at 3600 × g (4000 rpm) for five minutes in a Beckman J-6B centrifuge using a JS-4.2 rotor to sediment cell/tissue debris and nuclei. Supernatants were removed and then centrifuged for 30 minutes at 250,000 × g in a Beckman L80K ultracentrifuge using a Ti70.1 rotor. The Triton-soluble supernatant was used as the input for immunoprecipitations, and the Triton-insoluble fraction was resuspended in solubilization buffer supplemented with 1% deoxycholate and centrifuged for 30 minutes at 250,000 × g. To prepare membrane-enriched fractions for Fig S1B, the pulverized brains were resuspended in Membrane Buffer (300 mM sucrose, 75 mM NaCl, 20 mM EDTA, 10 mM EGTA, 100 μM PMSF, 500 μM benzamidine, 0.25 μM microcystin LR, 20 mM sodium β-glycerophosphate, 50 mM sodium fluoride, and protease inhibitor cocktail). Extracts were made and centrifuged as described above: supernatants were considered to be the cytosolic fraction, and the membrane-enriched pellets were resuspended in Solubilization Buffer to make a membrane-enriched fraction.

Immunoprecipitations from forebrain

The Triton-soluble fraction (1 to 1.5 ml) was precleared for 1 hour with protein-G Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and then incubated with 10 μg of either mouse monoclonal IgG_{2a} LTCC β subunit antibodies (Neuromab, Davis, Ca) or an affinity-purified polyclonal goat antibody that recognizes all CaMKII isoforms (McNeill & Colbran 1995). Control precipitations were performed using 10 μg of mouse (Jackson Labs, #015-000-003) or goat (Jiao *et al.* 2008) IgGs of mixed subtypes. After 1 hour, 10 μl of Protein-G agarose was added and the incubation continued for ~2 hours (or overnight for IPs from rat

brain) at 4°C. The resin was rinsed three times in 1 ml of solubilization buffer and bound proteins were analyzed by SDS-PAGE and western blotting with mouse antibodies to CaMKIIα (Affinity Bioreagents, Golden, Colorado) or LTCC subunits (α_1, β_1, and β_4: NeuroMab). A rabbit polyclonal β_2 antibody (a gift from Dr. Marlene Hosey) was used for the β_2 blot in Figure 1A.

Western Blotting

All samples were first separated in Tris-Glycine SDS-PAGE gels and then transferred to nitrocellulose membranes using standard methods. Membranes were blocked in 5% (w/v) milk in TTBS (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 0.1% (v/v) Triton-X-100), and then incubated with primary antibodies for 2 hours or overnight at 4°C. After washing 3 times for ~5 minutes each, membranes were incubated for 30 to 60 minutes at room temperature with horseradish peroxidase conjugated goat (Pierce, catalog number 31400), mouse (Promega, #W401B) or rabbit (Promega, #W4021) secondary antibody. Membranes were washed at least four times each for ~10 minutes and developed using enhanced chemiluminescence (Perkin-Elmer, Shelton, CT).

GST and His-tagged fusion protein expression and purification

Glutathione *S*-transferase (GST) and His-tagged fusion proteins were expressed in BL21-DE3 Gold *Escherichia coli* bacteria and purified using glutathione-agarose (Sigma, St Louis, MI) or His-Select Nickel Affinity Gel (Sigma), according to the manufacturers' protocol. Protein concentrations were determined by Bradford Assay (BioRad, Hercules, CA) or BCA assay (Pierce, Rockford, IL) using bovine serum albumin as a standard and validated by resolving proteins in SDS-PAGE gels and Coomassie-Blue staining.

CaMKII purification and autophosphorylation

Recombinant mouse CaMKIIα purified from baculovirus-infected Sf9 insect cells was autophosphorylated at Thr286 using ATP or [γ-^{32}P]ATP, essentially as described previously (McNeill & Colbran 1995). Briefly, CaMKII (1 μM subunit) was incubated on ice with 50 mM Tris, pH 7.5, 10 mM magnesium acetate, 1.5 mM $CaCl_2$, 20 μM calmodulin, 40 μM ATP, 1 mM DTT for 1.5 minutes and the reaction was stopped using 15 mM EDTA. For ^{32}P-labeling, CaMKII (5 μM subunit) was incubated on ice with 50 mM Tris, pH 7.5, 2 mM magnesium acetate, 1.5 mM $CaCl_2$, 20 μM calmodulin, 20 μM [γ-^{32}P]ATP (40,000 cmp/pmol) and 2 mM DTT. After 1 minute, 5 μl of 100 mM ATP was added and the reaction was continued for an additional minute before it was stopped with 10 mM EDTA. The radioactive CaMKII was desalted on a Sephadex G-50 column and used in the plate-binding assay.

Glutathione-agarose cosedimentation assay

Thr286-autophosphorylated CaMKIIα was incubated with GST-I/II linker (or GST-AID) with or without His-tagged β_{2a} for one hour at 4°C in GST pull-down buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100; 0.4 ml total volume). Glutathione-agarose (10 μl packed resin) was added, and the incubation was continued for one hour. Resin was collected by gentle centrifugation (1 min at 1000 rpm) and washed three times in binding buffer. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and detected by Ponceau-S staining. Membranes were scanned and then the levels of all proteins were quantified by densitometry using ImageJ (NIH).

CaMKII plate-binding assays

GST or GST-I/II proteins (100 pmol) were diluted into 0.2 ml of plate-assay 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) and immobilized in glutathione-coated wells for

18–24 hours at 4°C. [^{32}P-T^{286}]CaMKII (100 pmol) was premixed with buffer (no His-β_{2a}) or 36–100 pmol of His-β proteins. The mixture was added to the rinsed glutathione-coated wells. After a 2-hour incubation at 4°C, unbound kinase and His-β subunits were rinsed and the bound [^{32}P-T^{286}]CaMKII was quantified by scintillation counting. In some cases, contents of the wells were eluted into SDS-PAGE loading buffer and analyzed by electrophoresis, western blotting and autoradiography.

Immunoprecipitations from HEK293 cells

HEK293 cells were cotransfected with cDNA encoding HA-$Ca_v1.2\alpha_1$, FLAG-β_{2a} (WT or L493A) and EGFP-CaMKIIα using Fugene 6 (Roche, Indianapolis, IN). After a 5-minute treatment in 10 μM A23187, HA-$Ca_v1.2\alpha_1$ was immunoprecipitated from Triton-soluble cell extracts using 10 μg of anti-HA antibodies (Vanderbilt Monoclonal Antibody Core). Immune complexes were western blotted using HA, FLAG or EGFP antibodies.

LTCC subunit phosphorylation in cells

In order to assess total phosphorylation of LTCC subunits, HEK293 cells (≈1×10^6 cells per well in a 6 well plate) were transfected with HA-$Ca_v1.2\alpha_1$, EGFP-CaMKIIα and FLAG-β_{2a} (WT or L493A) cDNA for 48 hours. Intracellular phosphate pools were depleted by rinsing once in phosphate-free DMEM (GIBCO) and then incubating in the same medium for 1 hour prior to addition of 0.42 mCi per well of ^{32}P-orthophosphate (PerkinElmer, Boston, MA) diluted in phosphate-free DMEM. After incubating for 4 hours at 37°C, radioactive medium was removed and the cells were rinsed twice in ice-cold PBS. The HA-$Ca_v1.2\alpha_1$ subunit was immunoprecipitated from cell lysates (see above) and analyzed by SDS-PAGE. TOTAL ^{32}P-phosphorylation of LTCC subunits was detected by autoradiography, and total protein levels of each subunit were determined by western blotting with antibodies to the HA and FLAG epitopes. ImageJ was used to quantify β_{2a} ^{32}P-phosphorylation and protein levels, which were each corrected for background determined by similarly quantifying signals in control immune complexes isolated from cells lacking β_{2a}. Total ^{32}P-phosphorylation of β_{2a} was then normalized to total β_{2a} protein levels in the LTCC immune complex.

For specific assessment of Thr498 phosphorylation in FLAG-β_{2a}, HA-$Ca_v1.2\alpha_1$ immune complexes were western blotted with anti-phospho-Thr286 CaMKII (Promega, Madison, WI) which we previously showed also recognizes the phosphorylated Thr498 residue in β_{2a} (Grueter et al. 2008). Immunoblots were quantitatively analyzed using ImageJ. Signal intensities for phospho-Thr498 and FLAG-β_{2a} were each corrected for backgrounds (estimated from control complexes isolated from cells lacking β_{2a}), and phospho-Thr498 signals were then normalized to β_{2a} signals in each LTCC immune complex.

Statistics

Statistical analyses (t-tests or analyses of variance (ANOVA) and post-hoc tests) were performed using GraphPad Prism. P values less than 0.05 were considered significant.

RESULTS

Association of CaMKII with forebrain LTCC complexes containing β_1 or β_2 subunits

LTCC α_1 subunits and a heterogeneous mixture of β subunits are expressed in the brain. In order to determine whether CaMKII associates with these channels, we probed CaMKII immune complexes isolated from rat forebrain extracts for LTCC subunits (Fig. 1A). CaMKII was substantially depleted from the supernatant, with a corresponding enrichment in the immune complexes. Control IgGs failed to deplete CaMKII from the supernatant: the weak detection of CaMKII in control IgG complexes, despite extensive washing, likely reflects the

abundance of CaMKIIα in forebrain. Thus, CaMKII was substantially and specifically enriched in CaMKII complexes. Initial experiments indicated that rat CaMKII complexes also contained VGCC β_1 and β_2 subunit variants using limited available quantities of a rabbit antibody to the β_2 subunit and a mouse β_1 subunit antibody (Fig. 1A). Immunoblotting the rat CaMKII complexes with a monoclonal antibody to the $Ca_V1.2\alpha_1$ subunit also specifically and reproducibly detected an ≈200 kDa protein (not shown). This ≈200 kDa protein and the β_1 subunit were also specifically enriched in mouse forebrain CaMKII immune complexes (not shown). However, although the $Ca_V1.2\alpha_1$ antibody specifically detected $Ca_V1.2\alpha_1$ subunits expressed in HEK293 cells (not shown), we had no independent verification that the neuronal CaMKII-associated ≈200 kDa protein was in fact the $Ca_V1.2\alpha_1$ subunit. Nevertheless, these data together suggest that at least the LTCC β_1 and β_2 subunits are specifically associated with neuronal CaMKII.

In order to further explore the existence of LTCC-CaMKII complexes in brain, we first tested the specificity of commercially available monoclonal antibodies to the β_1 and β_4 subunits. Each antibody specifically detected the respective bacterially-expressed isoform as shown by western blotting GST fusion proteins containing the full length amino acid sequences of all four β subunit isoforms, albeit with different sensitivity (Fig S1A). The antibodies were then used to probe extracts of HEK cells transfected to express recombinant β_1 or β_4 and subcellular fractions of mouse brain (Fig. S1B). Both antibodies recognized the appropriate isoform in HEK cell lysates and also recognized a closely migrating series of bands that were enriched in brain particulate/membrane fractions. These proteins had apparent molecular weights appropriate for a mixture of splice variants and/or post-translationally modified forms of the respective β subunit.

The mouse β_1 and β_4 subunit antibodies were then used to immunoprecipitate calcium channel complexes from a Triton-soluble fraction of mouse brain. Western blots demonstrated that the appropriate β subunit isoform was specifically enriched in each immune complex (Fig. 1B/C). The $Ca_v1.2\alpha_1$ subunit was substantially enriched in both immune complexes, though somewhat more so in β_4 complexes than in β_1 complexes. In contrast, CaMKIIα was specifically detected in β_1 immune complexes (Fig. 1B) but not in β_4 immune complexes (Fig. 1C). In combination, these data demonstrate that CaMKIIα associates with a subset of LTCCs containing β_1 and β_2 subunits, but not with LTCC complexes containing the β_4 isoform.

The β subunits differentially tether CaMKII to LTCC α_1 subunits in vitro

We then investigated the role of direct interactions between the $Ca_v1.2\alpha_1$ and β subunits and/or CaMKII in assembly of CaMKII-LTCC complexes. We previously showed that Thr287-autophosphorylated CaMKIIδ directly interacts with the β_{1b} and β_{2a} subunits with similar affinities (K_d ≈100 nM CaMKII subunit), but not with β_3 or β_4 subunits (Grueter et al. 2008). The GK domains in β subunits bind to I/II linkers of VGCC α_1 subunits with high but variable affinities (5–60 nM) (Leroy *et al.* 2005, Van Petegem *et al.* 2008). In addition, autophosphorylated CaMKII was previously shown to directly bind with similar efficacy to multiple intracellular domains of the $Ca_V1.2\alpha_1$ subunit *in vitro*, including the I/II linker (Hudmon et al. 2005, Lee et al. 2006), although affinities of these interactions were not determined. As a first step toward dissecting roles of these interactions in assembly of CaMKII-LTCC complexes, we investigated binding of Thr286-autophosphorylated CaMKIIα to the GST-tagged I-II linker domain of the $Ca_V1.2\alpha_1$ subunit (GST-I/II) in the absence or presence of purified hexahistidine (His)-tagged β_{1b}, β_{2a} and β_3 subunits (we were unable to purify His-tagged β_4). There was no detectable direct interaction with GST-I/II, but CaMKII associated with GST-I/II in the presence of His-β_{1b} or His-β_{2a}, but not in the presence of His-β_3 (Fig. 2A). Interestingly, while β_{1b} and β_{2a} directly interact with autophosphorylated CaMKII with a similar apparent K_d (Grueter et al. 2008), His-β_{1b} immobilized significantly less CaMKII on

GST-I/II than did His-β_{2a}. Since CaMKII immobilization on the I/II linker also depends on β subunit interactions with the I/II linker, proteins were solubilized from the glutathione wells and analyzed by immunoblotting. GST-I/II appeared to bind similar amounts of β_{2a} and β_3, but the interaction with β_{1b} appeared to be weaker (Fig. 2B). Across the three experiments, GST-I/II binding to His-β_{1b} and His-β_3 was 45±10% and 102±37% of the binding to His-β_{2a} (mean±sem: p=0.03 and p=0.97, respectively). Moreover, GST-I/II binding to His-β_3 in GST-cosedimentation analyses was 85±15% of the binding to His-β_{2a} (n=3; p=0.43), and CaMKIIα binding did not appear to affect the interaction of His-β_{2a} with the I/II linker (Fig. S2 and see below). Thus, CaMKII can be tethered to the I/II linker of $Ca_V1.2\alpha_1$ subunits by specific β subunit isoforms *in vitro*.

Mechanism of β_{2a}-dependent tethering of CaMKIIα to the $Ca_V1.2\alpha_1$ I/II linker

Since β_{2a} most strongly tethers CaMKII to the CaV1.2α1 I/II linker, and is essential for CaMKII-dependent regulation of native LTCCs, we used β_{2a} to explore mechanisms underlying β subunit-dependent tethering of CaMKIIα to $Ca_V1.2\alpha_1$ subunits. Although we were unable to detect direct interactions of activated CaMKII with the 120 amino acid long I/II linker domain in GST-I/II, it seemed possible that binding of β_{2a} to CaMKIIα might stabilize direct interactions of CaMKIIα with the I/II linker. Therefore, we performed experiments using a GST fusion protein containing the minimal 18 amino acid alpha-interacting domain (GST-AID) that is sufficient for binding to β subunits (Van Petegem et al. 2008). As expected, we failed to detect direct interaction of activated CaMKIIα with GST-AID, but His-β_{2a} was able to tether activated CaMKII to GST-AID (Fig. S3). Mutation of Trp470 to Ala in the I/II linker was shown to severely compromise β subunit binding (Butcher *et al.* 2006, Leroy et al. 2005, Van Petegem et al. 2008), and the W470A mutation essentially abrogated the interactions of both His-β_{2a} and activated CaMKII with GST-I/II (Fig. 3A). High affinity direct binding of activated CaMKIIα to β_{2a} is disrupted by Leu493 to Ala mutation (Grueter et al. 2008). The L493A mutation significantly reduced β_{2a}-dependent co-sedimentation of activated CaMKIIα with GST-I/II by about 60% (Fig. 3A/C) and with GST-AID by about 70% (Fig. S3). Notably, disruption of CaMKII binding with the L493A mutation had no detectable effect on the amount of β_{2a} that co-sedimented with GST-I/II (Fig. 3B) or with GST-AID (Fig. S3), further confirming that interactions with CaMKII have no effect on the binding of His-β_{2a} to the I/II linker.

We then used a more quantitative glutathione-coated multi-well plate-binding assay (see Methods) to further explore mechanisms underlying His-β_{2a}-dependent tethering of activated ^{32}P-labeled CaMKII to GST-I/II. Consistent with co-sedimentation assays, activated CaMKII efficiently bound to GST-I/II in the presence of His-β_{2a}, and omission His-β_{2a} reduced binding by over 100-fold (Fig. 3D). The His-β_{2a}-dependent binding of CaMKII to GST-I/II also was significantly compromised by L493A mutation of His-β_{2a} (≈50% reduction in binding). Moreover, a His-β_{2a}(410–505) protein which lacks the SH3 and GK domains and cannot bind to the AID (data not shown), but contains the CaMKII-binding domain (Grueter et al. 2008), failed to support the tethering of activated CaMKII to GST-I/II (Fig. 3D). Taken together, these findings show that the β_{2a} subunit can serve as an adaptor protein to tether CaMKII to the I/II linker domain of the α_1 subunit.

The β_{2a} subunit enhances CaMKII association with full-length $Ca_V1.2\alpha_1$

Previous co-immunoprecipitation studies showed that CaMKII associates with LTCC complexes in the presence of β_2 subunits (Hudmon et al. 2005, Lee et al. 2006), but the role of the β subunit in formation of this complex was not explored. In order to investigate whether β_{2a} serves as an adaptor protein, tethering CaMKII to LTCC complexes, we expressed EGFP-CaMKII in HEK293 cells in the absence or presence of HA-$Ca_v1.2\alpha_1$ subunits and WT or L493A mutated FLAG-β_{2a}. LTCC complexes were isolated by immunoprecipitation using

anti-HA antibodies and western blotted for all three proteins. HA-$Ca_V1.2\alpha_1$ subunits were not detected in cell extracts due to the relatively low expression levels, but were readily detected in HA-immune complexes. As expected due to known roles of β subunits in modulating surface expression and trafficking of VGCC α_1 subunits, co-expression of β_{2a} enhanced the levels of α_1 subunit expression. WT and L493A mutated FLAG-β_{2a} proteins were expressed at similar levels and induced a similar increase in α_1 subunit expression. (Note: HA-immune complexes from HEK293 cells that were not transfected to express FLAG-β_{2a} contained a weak, non-specific FLAG-immunoreactive protein of about 70 kDa). Total expression levels of EGFP-CaMKII were similar in all transfections. However, EGFP-CaMKII was only reliably detected in anti-HA immune complexes isolated from cells expressing WT FLAG-β_{2a}. Much lower levels of EGFP-CaMKII were detected in anti-HA immune complexes isolated from cells expressing L493A-mutated FLAG-β_{2a} or from cells that did not express FLAG-β_{2a} (Fig. 4). These data demonstrate that high affinity binding to the β_{2a} subunit is necessary for efficient assembly of CaMKII with the LTCC complex.

CaMKII binding to β_{2a} selectively enhances phosphorylation of β_{2a} at Thr498

LTCCs are phosphorylated at multiple sites in both the α_1 and β subunits (reviewed by (Dai et al. 2009)) and CaMKII-dependent regulation of $Ca_V1.2$ LTCCs has been linked to phosphorylation of the α_1 subunit at Ser1512 and Ser1570 (Lee et al. 2006) and of the β_{2a} subunit at Thr498 (Grueter et al. 2006). In order to test the hypothesis that CaMKII binding to β_{2a} modulates LTCC subunit phosphorylation, HA-$Ca_V1.2\alpha_1$, EGFP-CaMKII and/or FLAG-β_{2a} (WT or L493A) were co-expressed in HEK293 cells. After labeling cells with ^{32}P-orthophosphate (see Methods), LTCC complexes were immunoprecipitated from cell extracts for analysis by SDS-PAGE followed by autoradiography to detect ^{32}P-phosphorylated proteins and by western blotting, allowing normalization of ^{32}P-phosphorylation to amounts of LTCC subunits isolated. The ^{32}P-labeling of $Ca_V1.2\alpha_1$ in the absence of co-expressed β_{2a} and EGFP-CaMKII indicated that endogenous kinases significantly phosphorylate $Ca_V1.2\alpha_1$ subunits under these basal incubation conditions (Fig. 5A). The co-expression of EGFP-CaMKII had no consistent effect on ^{32}P-phosphorylation of $Ca_V1.2\alpha_1$ in the absence or presence of WT β_{2a}. The ^{32}P-phosphorylation of WT β_{2a} within LTCC complexes also was not significantly altered by expression of EGFP-CaMKII (Fig. 5B). Moreover, L493A mutation of β_{2a} had no significant impact on ^{32}P-phosphorylation of either the $Ca_V1.2\alpha_1$ or β_{2a} subunit (Fig. 5A/B). However, we confirmed that EGFP-CaMKII associated with LTCC complexes containing WT β_{2a} subunits (data not shown). Thus, total ^{32}P-phosphorylation of $Ca_V1.2\alpha_1$ and β_{2a} subunits in LTCC complexes did not appear to be affected by CaMKII association via β_{2a} under basal conditions.

Total ^{32}P-phosphorylation of proteins in ^{32}P-orthophosphate labeling experiments can report the phosphorylation of multiple sites in a target protein. However, β_{2a} can be phosphorylated at multiple serine and threonine residues by several kinases, including PKA (at Ser 458,478,479) and PKG (at Ser 496) (Gerhardstein *et al.* 1999, Yang *et al.* 2007). Therefore, we reasoned that changes in phosphorylation at a limited number of sites due to L493A mutation may be obscured by ^{32}P-phosphorylation at other sites in β_{2a}. We previously showed that the critical regulatory phosphorylation of β_{2a} at Thr498 can be detected using a phospho-Thr286 CaMKIIα antibody (due to the similarity of surrounding amino acid sequences) and that L493A mutation does not affect recognition of phospho-Thr498 in β_{2a} (Grueter et al. 2008). Therefore, we used the anti-phospho-Thr286 CaMKIIα antibody to specifically examine Thr498 phosphorylation in β_{2a} within LTCC complexes immunoprecipitated from transfected HEK293 cells. We detected a robust signal with a phospho-Thr286 antibody within LTCC complexes containing WT β_{2a} subunits and mutation of Thr498 in β_{2a} to Ala eliminated the signal (Fig. S4). Moreover, a similar robust phosphorylation of WT β_{2a} was detected when EGFP-CaMKIIα was replaced by untagged CaMKIIα (data not shown). Thus, we specifically

detected Thr498 phosphorylation of β_{2a} in the LTCC complex under these conditions, and not Thr286 phosphorylation of co-precipitating EGFP-CaMKIIα. Significantly, mutation of Leu493 to Ala to interfere with CaMKII targeting to LTCC complexes almost abolished the phosphorylation of β_{2a} at Thr498 (Fig. 5C/D). Collectively, these data show that CaMKII binding to β_{2a} is required for efficient phosphorylation of a key regulatory site (Thr498) in β_{2a} without affecting overall phosphorylation of multiple sites in the $Ca_V1.2\alpha_1$ or β_{2a} subunits.

DISCUSSION

LTCC facilitation by CaMKII can be important for physiological augmentation of cellular Ca^{2+} signals in response to hormones or growth factors (Gao et al. 2006, O-Uchi et al. 2005). However, excessive $Ca_V1.2$ LTCC activity, Ca^{2+} entry and CaMKII activation are critical events in the pathogenesis of cardiac arrhythmias and other forms of heart disease (reviewed in (Couchonnal & Anderson 2008, Bers 2008)). Similarly, hyper-activation of $Ca_V1.2$ LTCCs by CaMKII is implicated in Timothy Syndrome, a multi-organ human genetic disorder whose symptoms include mental retardation and cardiac disease (Splawski *et al.* 2004, Splawski *et al.* 2005, Erxleben et al. 2006, Thiel et al. 2008). Furthermore, the loss of dendritic spines from striatal medium spiny neurons following dopamine depletion in animal models of parkinsonism results from excessive $Ca_V1.3$ LTCC activation (Day *et al.* 2006), in parallel with CaMKII over-activation (Brown *et al.* 2005). Thus, understanding mechanistic interactions between CaMKII and LTCCs promises to provide insights into physiological and pathological processes in multiple tissues.

CaMKII targeting to neuronal LTCCs by the β subunits

Subcellular compartmentalization is emerging as a key feature of Ca^{2+} signaling (Parekh 2008). Colocalization and functional studies suggest that a proportion of CaMKII and LTCCs exist in the same subcellular compartments in cardiomyocytes and neurons and that the association of CaMKII with the LTCC complex appears to be essential for facilitation (see Introduction). Multiple forms of β subunit- and CaMKII-dependent LTCC facilitation have been observed in heterologous cells (Lee et al. 2006, Xiao et al. 1994) or reconstituted in isolated membrane patches (Dzhura et al. 2002, Grueter et al. 2006). This implies that core elements for CaMKII regulation of $Ca_V1.2$ LTCCs in cardiomyocytes are intrinsic to the channel and must exist in neurons. CaMKII was previously shown to co-immunoprecipitate with cardiac $Ca_V1.2$ LTCC complexes. The present findings are the first to show that CaMKII associates with $Ca_V1.2$ LTCCs in forebrain.

Association of CaMKII with LTCCs in forebrain appears to be controlled by the β subunit identity. Although $Ca_V1.2\alpha_1$ subunits were similarly enriched in both β_1 and β_4 subunit immune complexes, CaMKII was only detected in the β_1 subunit complex (Fig. 1). Our biochemical studies suggest that CaMKII will also associate with neuronal LTCCs containing the β_2 subunit but not those containing the β_3 subunit (Figs. 1A, 2,3). This selective association with $Ca_V1.2$ LTCCs containing the β_1 and β_2 subunit variants appears to be due to differential scaffolding/adaptor protein functions of the β subunits. The GK domain of the β subunits interacts with the AID of the α_1 subunit, whereas a C-terminal domain conserved in β_{1b} or β_{2a}, but not in β_3 or β_4, binds to CaMKII. The number and stability of CaMKII-LTCC complexes may be influenced by additional interactions, including membrane association of β_1 or the β_2 subunits via N-terminal lipid modifications (reviewed in (Dolphin 2003,Hidalgo & Neely 2007)), additional interactions of β subunits with N- and C-terminal domains of the $Ca_v1.2\alpha_1$ subunit (Zhang et al. 2005,Kanevsky & Dascal 2006,Cheng *et al.* 2007,Lao *et al.* 2008) and CaMKII interaction with other $Ca_v1.2\alpha_1$ binding partners such as α-actinin (Robison *et al.* 2005,Malik 2009). However, the GK-AID interaction appears to be fundamental for

creation of a stable scaffold that specifically targets CaMKII to LTCCs containing the β_{1b} or β_{2a} subunit.

Association of β subunits with LTCCs in brain

Differential functions of β subunit isoforms in controlling the activity and membrane expression of VGCCs are widely accepted (Birnbaumer et al. 1998, Colecraft *et al.* 2002, Dolphin 2003). These differences are emphasized by the embryonic lethality of global knockout of β_1 and β_2 subunit genes in mice (Gregg *et al.* 1996, Weissgerber *et al.* 2006), whereas animals lacking the β_3 and β_4 subunits are viable (Murakami *et al.* 2002, Berggren *et al.* 2004). Relative expression levels of the β subunit variants are thought to play a major role in determining the specificity of β subunit association with LTCC α_1 subunits (Scott *et al.* 1996, Pichler *et al.* 1997, McEnery *et al.* 1998, Vance *et al.* 1998, Dolphin 2003). In contrast to the predominant expression of LTCC β_2 isoforms in cardiomyocytes, a mixture of β isoforms are differentially expressed in a complex, developmentally-regulated and cell-specific manner in the brain. The CaMKII-binding β_1 and β_2 subunits are expressed mainly in hippocampus, striatum, cerebellum and photoreceptors (Mermelstein *et al.* 1999, Ludwig *et al.* 1997, Vance et al. 1998, McEnery et al. 1998) and predominate during early postnatal development in rodents (Vance et al. 1998, Dolphin 2003). However, by adulthood, the β_3 and β_4 subunit variants together comprise about 70% of total β subunits in forebrain (Ludwig et al. 1997, Vance et al. 1998, McEnery et al. 1998). Thus, CaMKII association with neuronal $Ca_V1.2$ LTCCs may be more prevalent during postnatal development and only in certain brain regions or cell types.

CaMKII anchoring and LTCC regulation

It is increasingly apparent that subcellular targeting of CaMKII via protein-protein interactions promotes the phosphorylation of specific substrate targets in intact cells (Tsui *et al.* 2005, Tsui & Malenka 2006). We previously reported that CaMKII-dependent facilitation of $Ca_V1.2$ LTCCs requires the phosphorylation of β_{2a} subunits at Thr498, and showed that CaMKII binds with high affinity ($K_d \approx 100$ nM CaMKII subunit) to the β_{1b} and β_{2a} subunits, but not the β_3 or β_4 subunits. However, the relationship(s) between protein-protein interactions, phosphorylation and facilitation was not investigated. The primarily β-subunit-dependent association of CaMKII with LTCCs may facilitate CaMKII interaction with additional domains on the α_1 subunit (Hudmon et al. 2005), and may enhance CaMKII phosphorylation of functionally relevant sites, such as Ser1512 or Ser1570 in the α_1 subunit (Lee et al. 2006) or Thr498 in the β_{2a} subunit (Grueter et al. 2006). It was initially surprising that total ^{32}P-phosphorylation of LTCC subunits was not affected by co-expression of EGFP-CaMKII or by L493A mutation of β_{2a} (Fig. 5). However, it is important to note that these experiments were performed under basal cell incubation conditions in which CaMKII is only partially active. The partially active CaMKII may be more selective for the preferred Thr498 site in β_{2a} *in situ* relative to other sites that can be phosphorylated *in vitro* by fully active kinase (Grueter et al. 2006). The substantial background of LTCC subunit phosphorylation at other sites by endogenous kinases (Fig. 5) also may limit our ability to detect CaMKII-dependent changes in ^{32}P-labeling. However, we demonstrated by immunoblotting with a phospho-specific antibody that CaMKII binding to β_{2a} facilitates Thr498 phosphorylation within LTCC complexes isolated by immunoprecipitation. Since phosphorylation of β_{2a} at Thr498 is required for CaMKII-dependent LTCC facilitation, the present findings provide a biochemical explanation for these actions of CaMKII.

Do β subunits target CaMKII to regulate other VGCCs?

The β subunit isoforms associate with most VGCC α_1 subunit variants. Interestingly, CaMKII facilitates $Ca_V1.3$ LTCCs by phosphorylating the α_1 subunit at Ser1486 (Gao et al. 2006) and

antagonizes the inactivation of $Ca_v2.1$ P/Q type VGCCs by binding within the α_1 C-terminal tail (Jiang *et al.* 2008). It will be important to investigate whether β subunits also play a role in these CaMKII-dependent effects. Unlike $Ca_v1.3$ and 2.1, $Ca_V3.2$ channels are not generally thought to associate with β subunits. Yet CaMKII enhances the activity of $Ca_v3.2$ T-type VGCCs, apparently via direct interaction of CaMKII with the II/III linker domain (Welsby *et al.* 2003). Thus, binding of CaMKII to the VGCC complex appears to be a common feature for CaMKII-dependent feedback regulation of these channels. The β_1 or β_2 subunits may have a preeminent function in some cases, but in other cases direct interactions with the α_1 subunit may play an important role. Moreover, it will be interesting to investigate whether association of CaMKII with LTCCs via selected β subunit variants plays a role in CaMKII-dependent cross talk between LTCCs and R-type channels in dendritic spines (Yasuda et al. 2003, Lee et al. 2009) and/or in CaMKII activation to mediate downstream signaling to the nucleus (Wheeler et al. 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CaMKII	Calcium/Calmodulin-dependent Protien kinase II
LTCC	L-type calcium channel
VGCC or Ca_v	Voltage-gated calcium channel
CaM	Calmodulin
GST	Glutathione-S-transferase
HA	Hemoagglutinin
EGFP	enhanced green fluorescent protein
GK	guanylyl kinase
SH3	src homology 3

References

Anderson ME, Braun AP, Schulman H, Premack BA. Multifunctional Ca2+/calmodulin-dependent protein kinase mediates Ca(2+)-induced enhancement of the L-type Ca2+ current in rabbit ventricular myocytes. Circ Res 1994;75:854–861. [PubMed: 7923631]

Berggren PO, Yang SN, Murakami M, et al. Removal of Ca2+ channel beta3 subunit enhances Ca2+ oscillation frequency and insulin exocytosis. Cell 2004;119:273–284. [PubMed: 15479643]

Bers DM. Calcium cycling and signaling in cardiac myocytes. Annu Rev Physiol 2008;70:23–49. [PubMed: 17988210]

Birnbaumer L, Qin N, Olcese R, Tareilus E, Platano D, Costantin J, Stefani E. Structures and functions of calcium channel beta subunits. J Bioenerg Biomembr 1998;30:357–375. [PubMed: 9758332]

Bossuyt J, Helmstadter K, Wu X, Clements-Jewery H, Haworth RS, Avkiran M, Martin JL, Pogwizd SM, Bers DM. Ca2+/calmodulin-dependent protein kinase IIdelta and protein kinase D overexpression

reinforce the histone deacetylase 5 redistribution in heart failure. Circ Res 2008;102:695–702. [PubMed: 18218981]

Bourinet E, Charnet P, Tomlinson WJ, Stea A, Snutch TP, Nargeot J. Voltage-dependent facilitation of a neuronal alpha 1C L-type calcium channel. EMBO J 1994;13:5032–5039. [PubMed: 7957069]

Brown AM, Deutch AY, Colbran RJ. Dopamine depletion alters phosphorylation of striatal proteins in a model of Parkinsonism. Eur J Neurosci 2005;22:247–256. [PubMed: 16029214]

Butcher AJ, Leroy J, Richards MW, Pratt WS, Dolphin AC. The importance of occupancy rather than affinity of CaV(beta) subunits for the calcium channel I-II linker in relation to calcium channel function. J Physiol 2006;574:387–398. [PubMed: 16627564]

Calin-Jageman I, Lee A. Ca(v)1 L-type Ca2+ channel signaling complexes in neurons. J Neurochem 2008;105:573–583. [PubMed: 18266933]

Catterall WA. Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 2000;16:521–555. [PubMed: 11031246]

Cheng X, Liu J, Asuncion-Chin M, Blaskova E, Bannister JP, Dopico AM, Jaggar JH. A novel Ca(V)1.2 N terminus expressed in smooth muscle cells of resistance size arteries modifies channel regulation by auxiliary subunits. J Biol Chem 2007;282:29211–29221. [PubMed: 17699517]

Colbran RJ. Targeting of calcium/calmodulin-dependent protein kinase II. Biochem J 2004;378:1–16. [PubMed: 14653781]

Colecraft HM, Alseikhan B, Takahashi SX, et al. Novel functional properties of Ca(2+) channel beta subunits revealed by their expression in adult rat heart cells. J Physiol 2002;541:435–452. [PubMed: 12042350]

Couchonnal LF, Anderson ME. The role of calmodulin kinase II in myocardial physiology and disease. Physiology (Bethesda) 2008;23:151–159. [PubMed: 18556468]

Dai S, Hall DD, Hell JW. Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol Rev 2009;89:411–452. [PubMed: 19342611]

Davare MA, Dong F, Rubin CS, Hell JW. The A-kinase anchor protein MAP2B and cAMP-dependent protein kinase are associated with class C L-type calcium channels in neurons. J Biol Chem 1999;274:30280–30287. [PubMed: 10514522]

Day M, Wang Z, Ding J, et al. Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. Nat Neurosci 2006;9:251–259. [PubMed: 16415865]

Dolphin AC. Beta subunits of voltage-gated calcium channels. J Bioenerg Biomembr 2003;35:599–620. [PubMed: 15000522]

Dzhura I, Wu Y, Colbran RJ, Balser JR, Anderson ME. Calmodulin kinase determines calcium-dependent facilitation of L-type calcium channels. Nat Cell Biol 2000;2:173–177. [PubMed: 10707089]

Dzhura I, Wu Y, Colbran RJ, Corbin JD, Balser JR, Anderson ME. Cytoskeletal disrupting agents prevent calmodulin kinase, IQ domain and voltage-dependent facilitation of L-type Ca2+ channels. J Physiol 2002;545:399–406. [PubMed: 12456820]

Erxleben C, Liao Y, Gentile S, Chin D, Gomez-Alegria C, Mori Y, Birnbaumer L, Armstrong DL. Cyclosporin and Timothy syndrome increase mode 2 gating of CaV1.2 calcium channels through aberrant phosphorylation of S6 helices. Proc Natl Acad Sci U S A 2006;103:3932–3937. [PubMed: 16537462]

Gao L, Blair LA, Salinas GD, Needleman LA, Marshall J. Insulin-like growth factor-1 modulation of CaV1.3 calcium channels depends on Ca2+ release from IP3-sensitive stores and calcium/calmodulin kinase II phosphorylation of the alpha1 subunit EF hand. J Neurosci 2006;26:6259–6268. [PubMed: 16763033]

Gerhardstein BL, Puri TS, Chien AJ, Hosey MM. Identification of the sites phosphorylated by cyclic AMP-dependent protein kinase on the beta 2 subunit of L-type voltage-dependent calcium channels. Biochemistry 1999;38:10361–10370. [PubMed: 10441130]

Gregg RG, Messing A, Strube C, et al. Absence of the beta subunit (cchb1) of the skeletal muscle dihydropyridine receptor alters expression of the alpha 1 subunit and eliminates excitation-contraction coupling. Proc Natl Acad Sci U S A 1996;93:13961–13966. [PubMed: 8943043]

Griffith LC, Lu CS, Sun XX. CaMKII, an enzyme on the move: regulation of temporospatial localization. Mol Interv 2003;3:386–403. [PubMed: 14993460]

Grueter CE, Abiria SA, Dzhura I, Wu Y, Ham AJ, Mohler PJ, Anderson ME, Colbran RJ. L-type Ca2+ channel facilitation mediated by phosphorylation of the beta subunit by CaMKII. Mol Cell 2006;23:641–650. [PubMed: 16949361]

Grueter CE, Abiria SA, Wu Y, Anderson ME, Colbran RJ. Differential regulated interactions of calcium/calmodulin-dependent protein kinase II with isoforms of voltage-gated calcium channel beta subunits. Biochemistry 2008;47:1760–1767. [PubMed: 18205403]

Hidalgo P, Neely A. Multiplicity of protein interactions and functions of the voltage-gated calcium channel beta-subunit. Cell Calcium 2007;42:389–396. [PubMed: 17629941]

Hudmon A, Schulman H, Kim J, Maltez JM, Tsien RW, Pitt GS. CaMKII tethers to L-type Ca2+ channels, establishing a local and dedicated integrator of Ca2+ signals for facilitation. J Cell Biol 2005;171:537–547. [PubMed: 16275756]

Jiang X, Lautermilch NJ, Watari H, Westenbroek RE, Scheuer T, Catterall WA. Modulation of CaV2.1 channels by Ca2+/calmodulin-dependent protein kinase II bound to the C-terminal domain. Proc Natl Acad Sci U S A 2008;105:341–346. [PubMed: 18162541]

Jiao Y, Robison AJ, Bass MA, Colbran RJ. Developmentally regulated alternative splicing of densin modulates protein-protein interaction and subcellular localization. J Neurochem 2008;105:1746–1760. [PubMed: 18248607]

Kamp TJ, Hu H, Marban E. Voltage-dependent facilitation of cardiac L-type Ca channels expressed in HEK-293 cells requires beta-subunit. Am J Physiol Heart Circ Physiol 2000;278:H126–136. [PubMed: 10644592]

Kanevsky N, Dascal N. Regulation of maximal open probability is a separable function of Ca(v)beta subunit in L-type Ca2+ channel, dependent on NH2 terminus of alpha1C (Ca(v)1.2alpha). J Gen Physiol 2006;128:15–36. [PubMed: 16801381]

Lao QZ, Kobrinsky E, Harry JB, Ravindran A, Soldatov NM. New Determinant for the CaVbeta2 subunit modulation of the CaV1.2 calcium channel. J Biol Chem 2008;283:15577–15588. [PubMed: 18411278]

Lee SJ, Escobedo-Lozoya Y, Szatmari EM, Yasuda R. Activation of CaMKII in single dendritic spines during long-term potentiation. Nature 2009;458:299–304. [PubMed: 19295602]

Lee TS, Karl R, Moosmang S, Lenhardt P, Klugbauer N, Hofmann F, Kleppisch T, Welling A. Calmodulin kinase II is involved in voltage-dependent facilitation of the L-type Cav1.2 calcium channel: Identification of the phosphorylation sites. J Biol Chem 2006;281:25560–25567. [PubMed: 16820363]

Leroy J, Richards MW, Butcher AJ, Nieto-Rostro M, Pratt WS, Davies A, Dolphin AC. Interaction via a key tryptophan in the I-II linker of N-type calcium channels is required for beta1 but not for palmitoylated beta2, implicating an additional binding site in the regulation of channel voltage-dependent properties. J Neurosci 2005;25:6984–6996. [PubMed: 16049174]

Ludwig A, Flockerzi V, Hofmann F. Regional expression and cellular localization of the alpha1 and beta subunit of high voltage-activated calcium channels in rat brain. J Neurosci 1997;17:1339–1349. [PubMed: 9006977]

Malik AZ, Hall D Duane, Shea Madeline, Hell W Johannes. Role of Calmodulin on the Binding of Alpha-Actinin and C-terminus of Cav1.2 Ca2+ Channel. Biophys J 2009;96:186a.

McEnery MW, Vance CL, Begg CM, Lee WL, Choi Y, Dubel SJ. Differential expression and association of calcium channel subunits in development and disease. J Bioenerg Biomembr 1998;30:409–418. [PubMed: 9758336]

McNeill RB, Colbran RJ. Interaction of autophosphorylated Ca2+/calmodulin-dependent protein kinase II with neuronal cytoskeletal proteins. Characterization of binding to a 190-kDa postsynaptic density protein. J Biol Chem 1995;270:10043–10049. [PubMed: 7730306]

Mermelstein PG, Foehring RC, Tkatch T, Song WJ, Baranauskas G, Surmeier DJ. Properties of Q-type calcium channels in neostriatal and cortical neurons are correlated with beta subunit expression. J Neurosci 1999;19:7268–7277. [PubMed: 10460233]

Merrill MA, Chen Y, Strack S, Hell JW. Activity-driven postsynaptic translocation of CaMKII. Trends Pharmacol Sci 2005;26:645–653. [PubMed: 16253351]

Murakami M, Fleischmann B, De Felipe C, et al. Pain perception in mice lacking the beta3 subunit of voltage-activated calcium channels. J Biol Chem 2002;277:40342–40351. [PubMed: 12161429]

O-Uchi J, Komukai K, Kusakari Y, Obata T, Hongo K, Sasaki H, Kurihara S. alpha1-adrenoceptor stimulation potentiates L-type Ca2+ current through Ca2+/calmodulin-dependent PK II (CaMKII) activation in rat ventricular myocytes. Proc Natl Acad Sci U S A 2005;102:9400–9405. [PubMed: 15964981]

Parekh AB. Ca2+ microdomains near plasma membrane Ca2+ channels: impact on cell function. J Physiol 2008;586:3043–3054. [PubMed: 18467365]

Pichler M, Cassidy TN, Reimer D, Haase H, Kraus R, Ostler D, Striessnig J. Beta subunit heterogeneity in neuronal L-type Ca2+ channels. J Biol Chem 1997;272:13877–13882. [PubMed: 9153247]

Robison AJ, Bass MA, Jiao Y, MacMillan LB, Carmody LC, Bartlett RK, Colbran RJ. Multivalent interactions of calcium/calmodulin-dependent protein kinase II with the postsynaptic density proteins NR2B, densin-180, and alpha-actinin-2. J Biol Chem 2005;280:35329–35336. [PubMed: 16120608]

Scott VE, De Waard M, Liu H, Gurnett CA, Venzke DP, Lennon VA, Campbell KP. Beta subunit heterogeneity in N-type Ca2+ channels. J Biol Chem 1996;271:3207–3212. [PubMed: 8621722]

Splawski I, Timothy KW, Decher N, Kumar P, Sachse FB, Beggs AH, Sanguinetti MC, Keating MT. Severe arrhythmia disorder caused by cardiac L-type calcium channel mutations. Proc Natl Acad Sci U S A 2005;102:8089–8096. [PubMed: 15863612]

Splawski I, Timothy KW, Sharpe LM, et al. Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. Cell 2004;119:19–31. [PubMed: 15454078]

Thiel WH, Chen B, Hund TJ, Koval OM, Purohit A, Song LS, Mohler PJ, Anderson ME. Proarrhythmic defects in Timothy syndrome require calmodulin kinase II. Circulation 2008;118:2225–2234. [PubMed: 19001023]

Tsui J, Inagaki M, Schulman H. Calcium/calmodulin-dependent protein kinase II (CaMKII) localization acts in concert with substrate targeting to create spatial restriction for phosphorylation. J Biol Chem 2005;280:9210–9216. [PubMed: 15582994]

Tsui J, Malenka RC. Substrate localization creates specificity in calcium/calmodulin-dependent protein kinase II signaling at synapses. J Biol Chem 2006;281:13794–13804. [PubMed: 16551613]

Van Petegem F, Clark KA, Chatelain FC, Minor DL Jr. Structure of a complex between a voltage-gated calcium channel beta-subunit and an alpha-subunit domain. Nature 2004;429:671–675. [PubMed: 15141227]

Van Petegem F, Duderstadt KE, Clark KA, Wang M, Minor DL Jr. Alanine-Scanning Mutagenesis Defines a Conserved Energetic Hotspot in the CaV[alpha]1 AID-CaV[beta] Interaction Site that Is Critical for Channel Modulation. Structure 2008;16:280–294. [PubMed: 18275819]

Vance CL, Begg CM, Lee WL, Haase H, Copeland TD, McEnery MW. Differential expression and association of calcium channel alpha1B and beta subunits during rat brain ontogeny. J Biol Chem 1998;273:14495–14502. [PubMed: 9603963]

Weissgerber P, Held B, Bloch W, Kaestner L, Chien KR, Fleischmann BK, Lipp P, Flockerzi V, Freichel M. Reduced cardiac L-type Ca2+ current in Ca(V)beta2−/− embryos impairs cardiac development and contraction with secondary defects in vascular maturation. Circ Res 2006;99:749–757. [PubMed: 16946137]

Welsby PJ, Wang H, Wolfe JT, Colbran RJ, Johnson ML, Barrett PQ. A mechanism for the direct regulation of T-type calcium channels by Ca2+/calmodulin-dependent kinase II. J Neurosci 2003;23:10116–10121. [PubMed: 14602827]

Wheeler DG, Barrett CF, Groth RD, Safa P, Tsien RW. CaMKII locally encodes L-type channel activity to signal to nuclear CREB in excitation-transcription coupling. J Cell Biol 2008;183:849–863. [PubMed: 19047462]

Wu X, Zhang T, Bossuyt J, et al. Local InsP3-dependent perinuclear Ca2+ signaling in cardiac myocyte excitation-transcription coupling. J Clin Invest 2006;116:675–682. [PubMed: 16511602]

Wu Y, MacMillan LB, McNeill RB, Colbran RJ, Anderson ME. CaM kinase augments cardiac L-type Ca2+ current: a cellular mechanism for long Q-T arrhythmias. Am J Physiol 1999;276:H2168–2178. [PubMed: 10362701]

Xiao RP, Cheng H, Lederer WJ, Suzuki T, Lakatta EG. Dual regulation of Ca2+/calmodulin-dependent kinase II activity by membrane voltage and by calcium influx. Proc Natl Acad Sci U S A 1994;91:9659–9663. [PubMed: 7937825]

Yang L, Liu G, Zakharov SI, Bellinger AM, Mongillo M, Marx SO. Protein kinase G phosphorylates Cav1.2 alpha1c and beta2 subunits. Circ Res 2007;101:465–474. [PubMed: 17626895]

Yasuda R, Sabatini BL, Svoboda K. Plasticity of calcium channels in dendritic spines. Nat Neurosci 2003;6:948–955. [PubMed: 12937422]

Yuan W, Bers DM. Ca-dependent facilitation of cardiac Ca current is due to Ca-calmodulin-dependent protein kinase. Am J Physiol 1994;267:H982–993. [PubMed: 8092302]

Zhang R, Dzhura I, Grueter CE, Thiel W, Colbran RJ, Anderson ME. A dynamic alpha-beta inter-subunit agonist signaling complex is a novel feedback mechanism for regulating L-type Ca2+ channel opening. FASEB J 2005;19:1573–1575. [PubMed: 15994413]

Zhang T, Kohlhaas M, Backs J, et al. CaMKIIdelta isoforms differentially affect calcium handling but similarly regulate HDAC/MEF2 transcriptional responses. J Biol Chem 2007;282:35078–35087. [PubMed: 17923476]

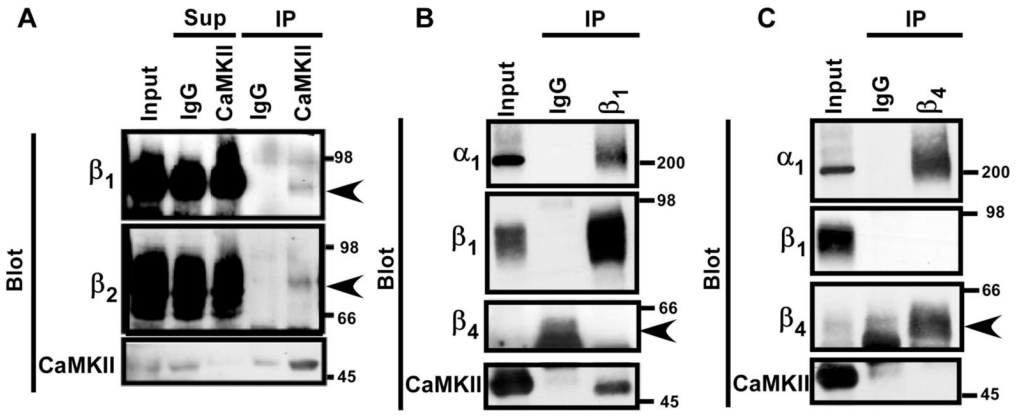

Figure 1. CaMKII associates with LTCC subunits in brain

Triton-soluble extracts of rat (**A**) or mouse (**B-C**) forebrains were immunoprecipitated using antibodies to CaMKII (**A**), β_1 (**B**) or β_4 (**C**) or control IgG. The immune complexes were western blotted for the indicated calcium channel subunits and CaMKII. These data are representative of 2 (**A**) or 4 (**B**/**C**) experiments.

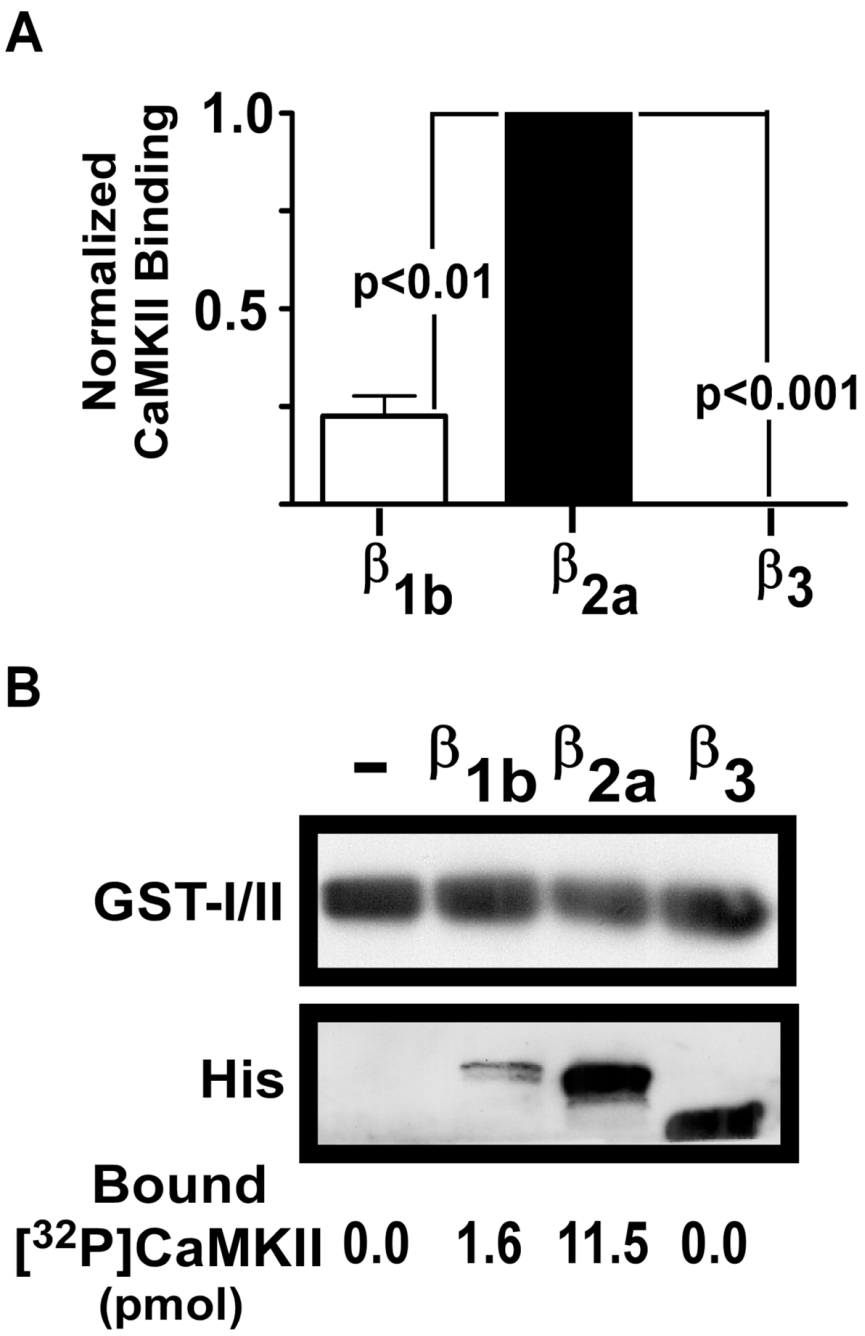

Figure 2. β subunits selectively anchor CaMKII to the $Ca_v1.2\alpha_1$ I/II linker *in vitro*

(**A**) GST-I/II (100 pmol) immobilized in glutathione-coated wells was incubated with a mixture of [^{32}P-T^{286}]CaMKIIα (100 pmol) and either His-β_1, His-β_{2a} or His-β_3 (36 pmol) in the presence of EDTA to prevent protein phosphorylation. Amounts of bound [^{32}P-T^{286}] CaMKIIα were quantified by scintillation counting and normalized to binding in the presence of β_{2a}. The graph plots data from 3 experiments (mean±sem) analyzed by ANOVA followed by Newman-Keuls multiple comparisons test. (**B**) Proteins were eluted from the glutathione-coated wells with SDS and western blotted with anti-GST or anti-His antibodies. The blots are representative of the three experiments, and [^{32}P-T^{286}]CaMKIIα binding in this specific experiment is shown below (mean of duplicates).

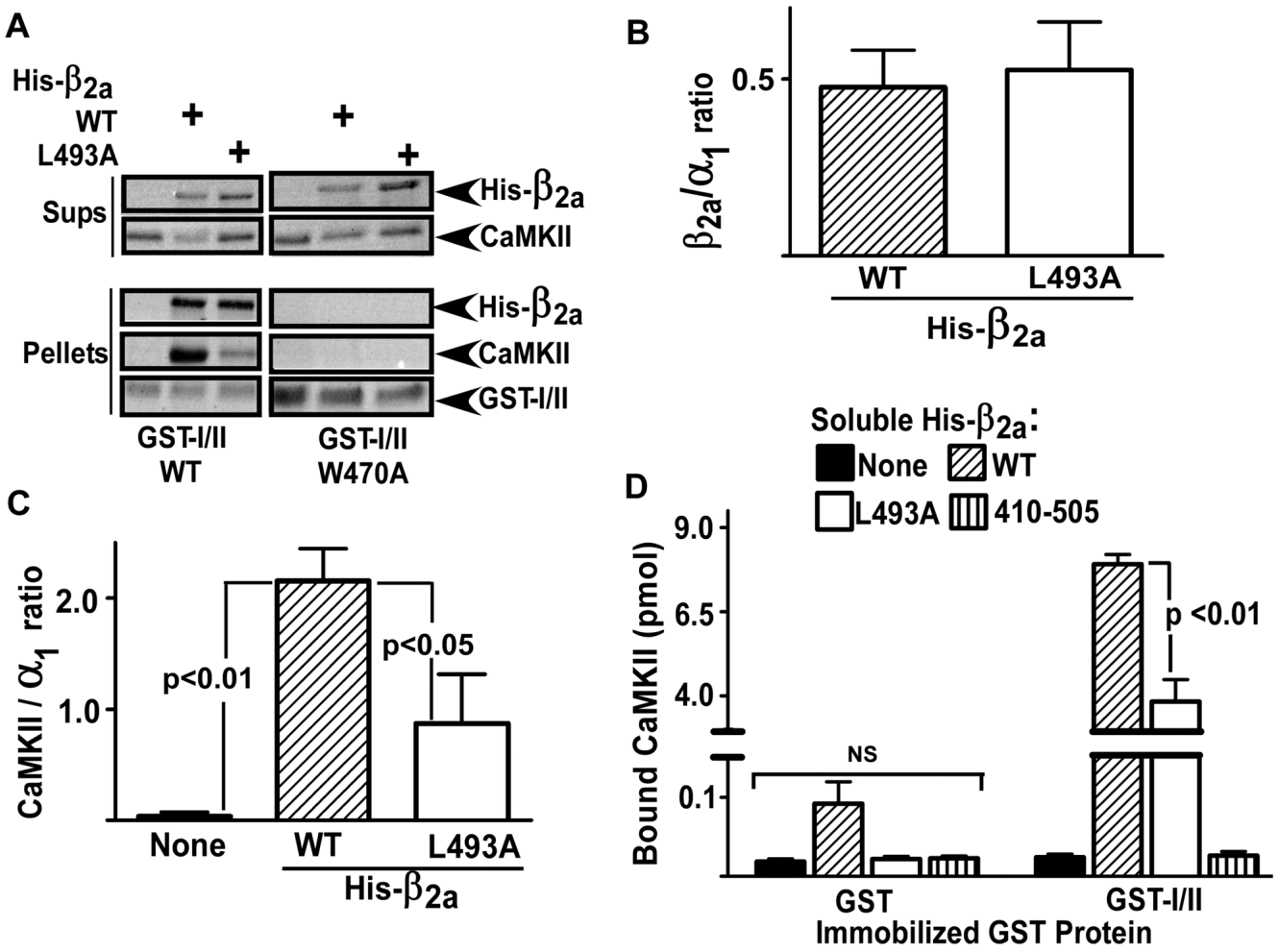

Figure 3. Molecular determinants of CaMKII anchoring to the $Ca_v1.2\alpha_1$ I/II linker

(A). Thr286-autophosphorylated CaMKIIα (100 pmol) was incubated with GST-I/II (WT or W470A) (100 pmol) with and without His-tagged β_{2a} (WT or L493A) (100 pmol). Complexes were isolated on glutathione agarose and the bound (pellets) and unbound (sups) proteins were detected by Ponceau-S staining. (**B)** Quantification (mean±sem, n=3) of β_{2a}(WT) and β_{2a}(L493A) binding to GST-I/II. **(C)** Quantification (mean±sem, n=3) of CaMKIIα binding to GST-I/II: data were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparisons test. **(D)** GST or GST-I/II (100 pmol) were immobilized in glutathione-coated wells and incubated with a mixture of [^{32}P-T^{286}]CaMKIIα (100 pmol) and either His-β_{2a} (WT or L493A) or His-β_{2a}(410–505) (100 pmol) in the presence of EDTA. Bound [^{32}P-T^{286}] CaMKIIα was quantified by scintillation counting. Data (mean±sem, n=3) were analyzed by two-way ANOVA with Bonferroni post-test.

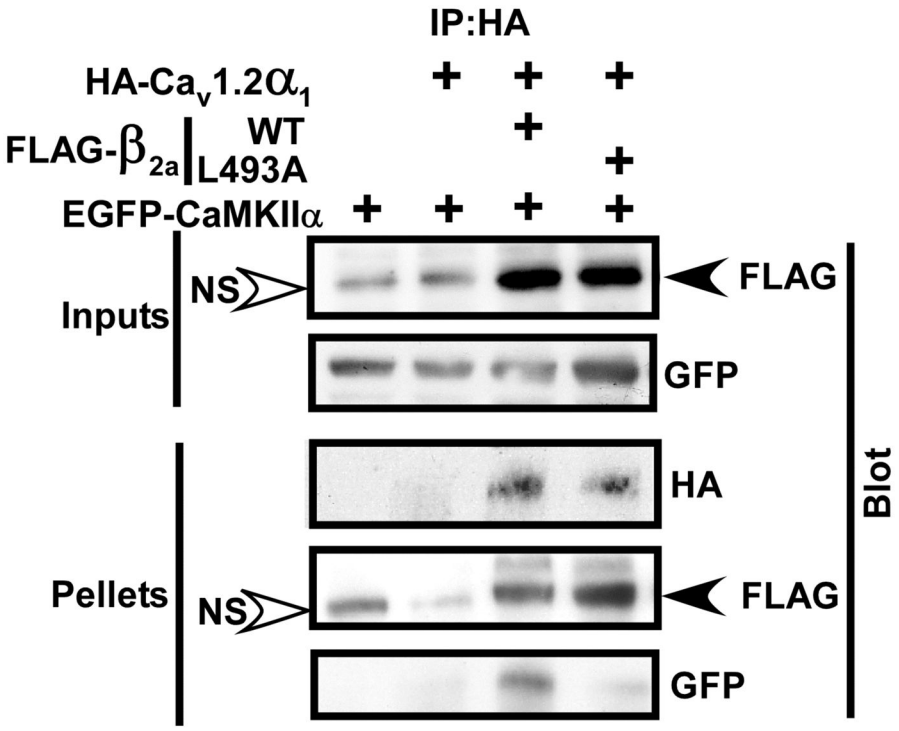

Figure 4. The β_{2a} subunit targets CaMKII to $Ca_v1.2\alpha_1$ subunits in heterologous cells

HEK293 cells co-expressing EGFP-CaMKIIα, HA-$Ca_v1.2\alpha_1$ and/or FLAG-β_{2a} (WT or L493A) as indicated were incubated for 5 min with A23187 (10 μM). Triton-soluble fractions (Inputs) and anti-HA immune complexes (pellets) were western blotted for HA, FLAG, or EGFP. Unfilled arrowheads indicate a non-specific (NS) FLAG immunoreactive band often detected in HA-immune complexes isolated from cells lacking FLAG-β_{2a} expression that migrates slightly faster than the FLAG-β_{2a} (filled arrowheads). These data are representative of 3 independent experiments.

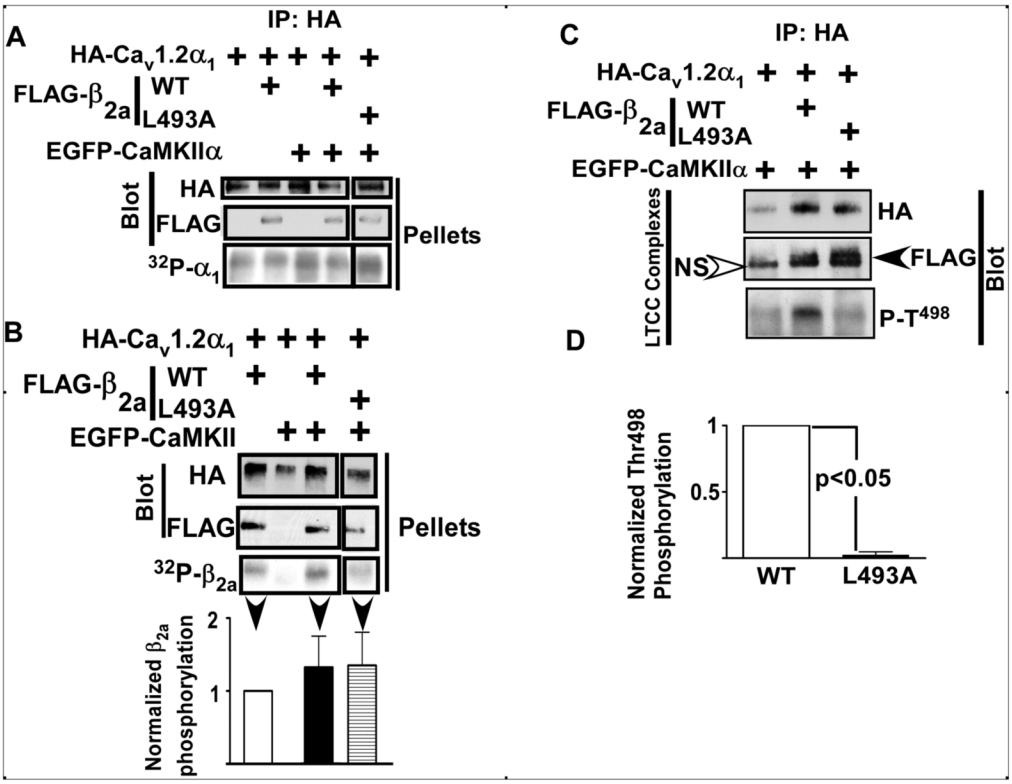

Figure 5. CaMKII binding does not affect total phosphorylation of $Ca_v1.2\alpha_1$ and β_{2a} but enhances β_{2a} phosphorylation at Thr498

LTCC complexes were immunoprecipitated from lysates of HEK293 cells co-expressing HA-$Ca_v1.2\alpha_1$, EGFP-CaMKIIα and FLAG-β_{2a} (WT or L493A) using antibodies to the HA epitope. In panels A and B the cells were labeled with ^{32}P-orthophosphate prior to lysis (see Methods). A. Anti-HA immune complexes were analyzed by SDS-PAGE and autoradiography to detect $Ca_v1.2\alpha_1$ phosphorylation, and western blotted as indicated. The data are representative of 4 independent experiments. B. Anti-HA immune complexes were analyzed by SDS-PAGE and autoradiography to detect phosphorylation of β_{2a} and were western blotted as indicated. The ^{32}P-phosphate incorporation was quantified and normalized to phosphorylation of wild-type β_{2a} in the absence of EGFP-CaMKIIα (mean±sem, n=4). C. Anti-HA immune complexes were analyzed by western blotting as indicated: blots are representative of 3 similar experiments. The unfilled arrowhead indicates a non-specific (NS) FLAG immunoreactive band detected in HA-immune complexes isolated from cells lacking FLAG-β_{2a} expression that migrates slightly faster than FLAG-β_{2a} (filled arrowhead). D. Quantification of Thr498 phosphorylation of wild-type/mutated FLAG-β_{2a} in anti-HA immune complexes, normalized to phosphorylation of wild-type β_{2a} (mean±sem, n=3). Significance was assessed using a one-sample t-test. CaMKII associates with $Ca_V1.2$ L-type calcium channels via selected β subunits to enhance regulatory phosphorylation.