New Determinant for the \( \text{CaV}_1.2 \) Subunit Modulation of the \( \text{CaV}_1.2 \) Calcium Channel*

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Ca\( \beta \) subunits support voltage gating of \( \text{CaV}_1.2 \) calcium channels and play important role in excitation-contraction coupling. The common central membrane-associated guanylate kinase (MAGUK) region of Ca\( \beta \) binds to the \( \alpha \)-interaction domain (AID) and the IQ motif of the pore-forming \( \alpha_{1C} \) subunit, but these two interactions do not explain why the cardiac Ca\( \beta \_2 \) subunit splice variants differentially modulate inactivation of Ca\( ^{2+} \) currents (\( I_{\text{Ca}} \)). Previously we described \( \beta_{2\Delta_{\alpha}} \), a functionally active splice variant of human Ca\( \beta \_2 \), lacking MAGUK. By deletion analysis of \( \beta_{2\Delta_{\alpha}} \) we have now identified a 41-amino acid C-terminal essential determinant (\( \beta_{2CED} \)) that stimulates \( I_{\text{Ca}} \) in the absence of Ca\( \beta \) subunits and conveys a \( +20\)-mV shift in the peak of the \( I_{\text{Ca}} \)-voltage relationship. The \( \beta_{2CED} \) is targeted by \( \alpha_{1C} \) to the plasma membrane, forms a complex with \( \alpha_{1C} \) but does not bind to AID. Electrophysiology and binding studies point to the calmodulin-interacting LA/IQ region in the \( \alpha_{1C} \) subunit C terminus as a functionally relevant \( \beta_{2CED} \) binding site. The \( \beta_{2CED} \) interacts with LA/IQ in a Ca\( ^{2+} \)- and calmodulin-independent manner and need LA, but not IQ, to activate the channel. Deletion/mutation analyses indicated that each of the three Ca\( \beta_{2\Delta_{\alpha}/\alpha_{1C}} \) interactions is sufficient to support \( I_{\text{Ca}} \). However, \( \beta_{2CED} \) does not support Ca\( ^{2+} \)-dependent inactivation, suggesting that interactions of MAGUK with AID and IQ are crucial for Ca\( ^{2+} \)-induced inactivation. The \( \beta_{2CED} \) is conserved only in Ca\( \beta \_2 \) subunits. Thus, \( \beta_{2CED} \) constitutes a previously unknown integrative part of the multifactorial mechanism of Ca\( \beta \_2 \)-subunit differential modulation of the \( \text{CaV}_1.2 \) calcium channel that in \( \beta_{2\Delta_{\alpha}} \) occurs without MAGUK.

Voltage-gated \( \text{CaV}_1.2 \) calcium channels couple membrane depolarization to excitation in a wide variety of cells. The voltage gating, or membrane potential-dependent opening and closing of a channel, is associated with conformational changes in the pore-forming (\( \alpha_1 \)) subunit (1). \( \text{CaV}_1.2 \) channels require auxiliary \( \alpha_2\delta \) and \( \beta \) (Ca\( \beta \)) subunits to integrate the functional channel into the plasma membrane (PM) and facilitate voltage-gating of the current (2). How Ca\( \beta \) subunits mediate these functions and what are the Ca\( \beta \)-specific determinants are important questions to be answered. Members of the Ca\( \beta \) family are structurally divergent. Four different Ca\( \beta \) subunit genes code for \( \beta_1 \)-\( \beta_4 \) subunit variants, some of which are alternatively spliced. Cytosolic Ca\( \beta \) subunits bind to the 18-amino acid \( \alpha_1 \)-interaction domain (AID) of the cytoplasmic linker between internal repeats I and II of the pore-forming \( \alpha_1 \) subunit (Fig. 1), stimulate the Ca\( ^{2+} \) channel current (\( I_{\text{Ca}} \)), and shift the current-voltage (\( I-V \)) curve to more negative voltages (3, 4). The AID is conserved between the \( \text{CaV}_1 \) and \( \text{CaV}_2 \) subfamilies of Ca\( ^{2+} \) channels. It is located in close proximity to the transmembrane segment IS6 that is a part of the pore domain (5) implicated in voltage-dependent inactivation of the channel (6, 7). A common central region of Ca\( \beta \) subunits has structural similarity with the membrane-associated guanylate kinase (MAGUK) domain (6), holding potential (7). When co-expressed with the \( \alpha_{1C} \) subunit, the Ca\( \beta \) MAGUK domain increased Ba\( ^{2+} \) current (\( I_{\text{Ba}} \)) amplitude and shifted the steady-state activation (9). Confirming tight binding of the central Ca\( \beta \) domain to the \( \alpha_{1C} \) subunit, diffraction studies revealed structural patterns that were implicated in interaction with the AID (10–12). However, variant-specific regulatory properties of Ca\( \beta \) appear to be AID-independent. Although different Ca\( \beta \) subunits have MAGUK, they modulate Ca\( ^{2+} \) channels with individual characteristic variations. For example, the primary cardiac \( \beta_{2a} \) subunit did not fully substitute the \( \beta_{1a} \) subunit in skeletal muscle EC coupling although it restored activation of \( I_{\text{Ca}} \) and gating of Ca\( ^{2+} \) transients (13). Unlike other Ca\( \beta \) subunits, \( \beta_{2a} \) endows the distinct cardiac phenotype by not supporting facilitation of the Ca\( ^{2+} \) channel current by a depolarizing prepulse (14). This general picture was further detailed by FRET microscopy combined with patch clamp that demonstrated differential voltage-dependent rearrangement of Ca\( \beta \) subunits via \( \alpha_1 \)\( \forall \) via \( \alpha_{1C} \) subunit N terminus (15). Unlike the Ca\( \beta_{1a} \) subunit, Ca\( \beta_{2a} \) exhibited no such mobility. These and other findings show that a number of Ca\( \beta \) functions do not rely on AID as a main site of regulation and may involve other determinants (16, 17). Thus, identification of functional motifs that are unique for different Ca\( \beta \) subunits may give an important insight into the functional specificity of the Ca\( \beta \)-dependent modulation. One feasible approach is to explore the naturally occurring Ca\( \beta \) splice variants (18). In line with this was the discovery of two new functionally active small

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The abbreviations used are: PM, plasma membrane; Ab, antibody; AID, \( \alpha_1 \)-interaction domain; anti-LC, anti-Living Color Ab to EGF; \( \beta_2CED \), C-terminal essential determinant of the Ca\( \beta_2 \) subunit; CaM, calmodulin; CDI, Ca\( ^{2+} \)-dependent inactivation; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; IP, immunoprecipitation; MAGUK, membrane-associated guanylate kinase; \( V_p \), holding potential; \( V_h \), test potential; SH, Src homology domain; HEK, human embryonic kidney.
splice variants of the human cardiac β2 subunit lacking the central domain (19). These β2f and β2ag subunits share a 153-amino acid distal C-terminal region common to all known "large" Caβ2 subunits (β2ag–β2a) (20) suggesting that this region may have a role of an essential Cav2 determinant. Our attention to this region of the β2 subunit was stimulated by the finding that β2ag supports IcaL on co-expression with α1c and α2δ in Caβ-free COS1 cells. Because large and small Caβ2 splice variants convey sharply different inactivation kinetics, it seems apparent that, in addition to MAGUK, there is a C-terminal determinant (defined here as β2CED) that is common only to Caβ2 subunits and thus may contribute to the Caβ2-specific tuning of the channel modulation by large Caβ2 subunits. In the case of the small Caβ2 subunits, β2CED may play the key regulatory role. This intriguing possibility prompted us to locate β2CED and characterize the properties of β2CED-modulated Ca2+ channels that rely on β2CED-dependent, MAGUK-independent modulation.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology, β2ag Deletion Mutants—Cloning of β2ag (AY675092), subcloning into the pcDNA3 vector, and the 5′-terminal fusion of ECFP have been described previously (19). To create ECFP-labeled β2ag deletion mutants, the general strategy was to generate the deletion mutants by PCR and then replace the β2ag open reading frame in 5′-ECFP-β2ag-pcDNA3 with a deletion mutant at the 5′-XhoI/Apal-3′ sites. The PCR were performed with β2ag as template using the following primer pairs with 5′-XhoI linker (sense) and 3′-Apal-TGA linker (antisense): 5′-cgcctgggtgtctctggagggagagacc-3′ (sense) and 5′-gggctgctagcatgggtgctgtttcaggaag-3′ (antisense) for β2ag(1–82); 5′-gctgctagcatgggtgctgtttcaggaag-3′ (sense) and 5′-gggctgctagcatgggtgctgtttcaggaag-3′ (antisense) for β2ag(83–123); 5′-cggcgtgctagcatgggtgctgtttcaggaag-3′ (sense) and 5′-gggctgctagcatgggtgctgtttcaggaag-3′ (antisense) for β2ag(124–164). To create the deletion mutant 5′-ECFP-β2ag(83–164)-pcDNA3, 5′-ECFP-β2ag-pcDNA3 was cut by XhoI, filled-in to a blunt end, cleaved by PmlI, and then the 6.2-kb fragment was self-ligated. To generate the unlabelled β2ag(124–165), a PCR product was amplified using the NotI linker/Kozak primer (sense) 5′-ccgcgtgctagcatgggtgctgtttcaggaag-3′ and the Apal primer/linker (antisense) 5′-gctgctgctagcatgggtgctgtttcaggaag-3′. The β2ag(124–165) PCR product was subcloned into 5′-ECFP-β2ag-pcDNA3 or β2ag-pcDNA3 plasmid at the NotI/Apal sites (replacing the β2ag cassette). To create the mVenus-labeled deletion mutants, the 5′-BsrGI/Apal-3′ inserts of the ECFP-labeled deletion mutants were subcloned into the mVenus-C1 vector (kindly provided by Dr. S. S. Vogel) cleaved at the same sites.

5′-ECFP-β2ag-pcDNA3 was prepared by ligation of the 5′-PshAI/BsmBl-3′ fragment of β2ag into the respective sites of plasmid 5′-ECFP-β2a-pcDNA3. The 5′-ECFP-β2agCED deletion construct in pcDNA3 was produced by substitution of 5′-ECFP-β2ag-pcDNA3 at the BsmBl-AvrII sites with the respective fragment of β2ag(83–123)-pcDNA3. The β2ag cDNA was amplified by reverse transcriptase-PCR from human heart mRNA with the sense 5′-ATGCCTTACAGACGCCCTTATAG-3′ and antisense 5′-GCTGTTAGTTATACAGAAGCCTTC-3′ primers as described earlier (19).

FLAGN-β2ag and FLAGN-β2agCED in pcDNA3 were produced by ligating the 5′-BsrGI (filled in with Klenow)/AvrII-3′ fragments of pECFPN-β2ag and pECFPN-β2agCED, respectively, into a FLAG-2AB-pcDNA3 vector (gift of Dr. Kuanghueih Chen) at the EcoRV and AvrII sites. To prepare mVenusN-β2ag and mVenusN-β2agCED in pcDNA3, the 5′-BsrGI/AvrII (filled in with Klenow)-3′ fragments of 5′-ECFP-β2ag-pcDNA3 and 5′-ECFP-β2agCED-pcDNA3, respectively, were ligated into the mVenus-C1 vector at the BsrGI and EcoRI (filled in with Klenow) sites.

mVenusN-I-IIAID was constructed by PCR amplification of the 418–455 fragment of the α1c,77-1-II linker (nucleotides 1252–1365 of the pHLC77 (z34815) open reading frame) in pcDNA3 followed by subcloning into the 5′-PseBl/EcoRI-3′ sites of the mVenus-C1 vector. The sense and antisense primers used were 5′-ccgctgctagcatgggtgctgtttcaggaag-3′ and 5′-ccgctgctagcatgggtgctgtttcaggaag-3′ as sense and antisense primers, respectively. mVenusN-I-IIAID was created in the same way except that α1c,77AIDM was used as the template for PCR. Bioin2N-I-IIAID in pcDNA6 was prepared with a Zero Blunt TOPO PCR cloning kit (Invitrogen) and a pcDNA™ 6 BioEase Gateway Biotinylation System (Invitrogen) according to the manufacturer’s instructions using α1c,77-pcDNA3 as template, 5′-cacatggtggctgtttcaggaag-3′ and 5′-ccgctgctagcatgggtgctgtttcaggaag-3′ as sense and antisense primers, respectively.

To prepare FLAGN-LA/IQ (amino acids 1571–1636 of the α1c,77 I-II linker (nucleotides 1252–1365 of the pHLC77 (z34815) open reading frame) in pcDNA3, PCR was performed with α1c,77-pcDNA3 as template, 5′-gtatggagccggtgagggagagacc-3′ and 5′-ccgctgctagcatgggtgctgtttcaggaag-3′ as sense and antisense primers, respectively; the PCR product was then subcloned into a FLAG-2AB-pcDNA3 vector at the HindIII/Apal sites. FLAGN-α1c,77-pcDNA3 was created by replacement of the
5’-Ndel/HindIII-3’ fragment of α1C,77-pcDNA3 with the respective fragment from FLAG-2AB-pcDNA3.

The AID mutant α1C,77AIDM (D433A,G436A,Y437A,W440A) was generated by a “two-step” PCR site-directed mutagenesis. Briefly, two PCR fragments were produced with α1C,77-pcDNA3 as template and pairs of outer sense and mutagenesis antisense primers, and mutagenesis sense and outer antisense primers, respectively. The mutagenesis antisense and sense primers contained the desired mutated sequence and had 18 bases complementary to each other. The two fragments were fused together by denaturing, annealing, and Taq polymerase extension, the fused DNA product was then amplified by PCR with the outer primer pair. The outer sense and antisense primers were designed from the vector region 5’-ctataggagaccaagcttc-3’ and α1C,77 open reading frame 5’-CACCCTTCTTGCAGAACC3’ (1550 → 1531), respectively; the mutagenesis antisense and sense primers were 5’-ATCCAGGccGTTTTGAGAgCCTCTCAGCATGCTGTTT-3’ and 5’-CTCAAGGgcGCTTTGAGGcGACCGGGAAGAC3’ (sense) and 5’-CTGGAATACATTTGACGCCTTGAGGcCTGAGGc-3’ (sense) and 5’-ggccacgagcttcGAGGCCGCTCCTGACCAGGGC-3’ (antisense); the outer primers were 5’-CTGGAATACATTTGACGCCTTGAGGcCTGAGGc-3’ (sense) and 5’-ctataggagaccaagcttc-3’ (antisense). The mutagenesis antisense and sense primers were 5’-NsiI/XbaI-3’ and 5’-XbaI/NsiI-3’ respectively; the mutagenesis antisense and sense primers contained the desired mutated sequence and had 18 bases complementary to each other. The plasmid was cleaved with HindIII and ClaI and substituted as a standard cross-linking reaction (22, 23) by incubation with dithiobis(succinimidyl propionate) (1 mM) (Pierce) at room temperature for 30 min. Cross-linking was stopped by incubation of cells with 20 mM Tris-HCl (pH 7.5) for 15 min. Cells were lysed with a Cel-Lytic M lysis reagent (450 μl/plate, Sigma) containing a protease inhibitor mixture (Sigma, 1/100 dilution) supplemented with 1 mM phenylmethylsulfonyl fluoride. To ensure direct interaction, the microsomal fraction was used for co-IP experiments involving α1C. The 80-μl aliquots of total lysates were kept to verify the expression of each protein (see “input” on immunoblots). Co-IP was performed with the selected antibodies according to the manufacturer’s instructions. Briefly, co-IP with anti-FLAG antibody (Ab) was performed with 40 μl reaction of a monoclonal EZ ViewTM ANTI-FLAG® M2 affinity gel (Sigma) at 4°C overnight, and the immunoprecipitates were eluted by incubation with 10 μg of 3 × FLAG peptide (Sigma) in 100 μl of TBS solution (pH 7.4) at 4°C for 1 h. The co-IP with anti-LC Ab was performed with 5 μl of a Living Colors Full-Length A.V. polyclonal Ab (Clontech) using Protein A-agarose (Sigma) as carrier (overnight at 4°C), and the immunoprecipitates were eluted by boiling for 5 min at 95°C. Dithiobis(succinimidyl propionate) was cleaved by incubation of the co-immunoprecipitate and input samples with 5% β-mercaptoethanol at 100°C for 5 min or with 50 mM dithiothreitol at 37°C for 30 min (only for α1C) before SDS-PAGE. SDS-PAGE and immunoblotting with the indicated antibodies were performed according to standard protocols. The following primary antibodies were used: anti-FLAG M2 monoclonal Ab (2 μg/ml, Sigma) for the FLAG-tagged proteins, Living Colors Full-Length A.V. monoclonal Ab (0.5 μg/ml, Clontech) for the fluorescent tagged proteins, and streptavidin-horseradish peroxi-

**FIGURE 2. Characterization of expression of Ca2+ channels in COS1 cells.** A, endogenous α1C subunits are not detectable in COS1 cells by Western blotting. Lane 1, non-transfected COS1 cells. Lane 2, COS1 cells expressing the recombinant α1C,77/α1C,77δ/β2,δ channel. Immunoblot analysis was carried out with antibody against α1C (Chemicon). The position of the α1C subunit is marked on the left side; molecular mass (in kDa) is shown on the right side. B, representative trace of I_{Ca} generated in response to V_{app} = +30 mV applied from V_{0} = −90 mV to COS1 cells transfected by EYFPN-α1C,77 and α1C,77δ subunits in the absence of Ca δ subunits. No current was observed between 0 and +50 mV. C, superimposed traces of the maximal I_{Ca} through the EYFPN-α1C,77/α1C,77δ channel with β2,δ (trace 1) or ECFP_N-β2,δ (trace 2) recorded at V_{app} = +40 mV and normalized to the same amplitude. The α1C, α1C,77δ and β2,δ subunits were co-expressed in a 1:1:1 molar ratio. No significant difference in the kinetics of the currents was observed.
dase (1/1000 dilution, Invitrogen) for the biotin-tagged proteins. Nitrocellulose membrane (Invitrogen) was used for immunoblot analysis of α1C co-IP experiments and polyvinylidene difluoride membrane (Invitrogen) was used for all other studies.

Electrophysiology—The Effectene kit (Qiagen) was used for transfection of COS1 cells as described previously (15) under conditions optimized for a total amount of 0.2 μg of DNA per 35-mm Petri dish. Constructs were expressed in a 1:1 molar ratio. COS1 cells were grown on poly-D-lysine-coated coverslips (MatTek) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Whole-cell patch clamp recordings were performed as described (24) at 20–22 °C using the Axopatch 200B amplifier (Axon Instruments) 48–72 h after transfection. The extracellular bath solution contained (in mM): 100 NaCl, 20 BaCl2 or CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, adjusted to pH 7.4 with NaOH. Borosilicate glass pipettes (Kimax-51, Kimble Products) were fire-polished and showed a typical resistance of 3–6 megohms when filled with pipette solution containing (in mM): 110 CsCl, 5 MgATP, 10 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, 20 tetraethylammonium, 0.2 cAMP, and 20 HEPES, adjusted to pH 7.4 with CsOH (15). Voltage protocols were generated and data were digitized, recorded, and analyzed using pClamp 8.1 software (Axon Instruments). Test pulses were applied at 15-s intervals from the holding potential $V_h = -90$ mV. Currents were filtered at 1 kHz, sampled at 2.5–5 kHz, and corrected for leakage using an on-line P/4 subtraction protocol. At the end of experiments, channels were routinely tested for sensitivity of $I_{Ca}$ to the inhibition by the dihydropyridine blocker PN200-110 (see examples). $I-V$ curves were obtained by step depolarization to test potentials in the range of $-60$ to $+90$ mV (with 10-mV increments) applied from $V_h$. Steady-state inactivation curves were measured with conditioning pulses (1 s) applied from $V_h = -90$ mV up to $-60$ to $+40$ mV with 10-mV increments followed by a 100-ms test pulse. Peak current amplitudes were normalized to the maximal value. Averaged $I-V$ curves
were fit with the equation: 

\[ I = G_{\text{max}} (V - E_{\text{rev}})/(1 + \exp((V - V_{0.5, \text{act}})/k_{1, \text{v}})) \]

where \( G_{\text{max}} \) is maximum conductance, \( E_{\text{rev}} \) is reversal potential, \( V_{0.5, \text{act}} \) is the voltage at 50% of the current (I) activation, and \( k_{1, \text{v}} \) is the slope factor. Steady-state inactivation curves were fitted by a Boltzmann function:

\[ I = a + b(1 + \exp((V - V_{0.5, \text{in}})/k_{2, \text{v}})) \]

where \( V \) is the conditioning pulse voltage; \( V_{0.5, \text{in}} \) is the voltage at half-maximum of inactivation, \( k_{2, \text{v}} \) is a slope factor, and \( a \) and \( b \) are fractions of noninactivating and inactivating components of the current, respectively. To estimate the time constant \( \tau \) of inactivation, currents were fitted with the Chebyshev method according to the standard exponential function,

\[ I(t) = \sum_{i=1}^{n} I\text{i} e^{-t/\tau_i} + I_0 \quad \text{(Eq. 1)} \]

where \( I_i \) is the amplitude of the inactivating component of the current, \( \tau \) is the time constant of inactivation, and \( I_0 \) is the non-inactivating component of the current. Statistical values are given as mean ± S.E. Error bars in the figures are S.E., n, number of tested cells. Differences were considered significant if Student’s t test showed \( p < 0.05 \).

Imaging—Cells images were recorded with a 14-bit Hamamatsu digital camera C9100-12 mounted on the Nikon epifluorescent microscope TE200 (60 × 1.2 N.A. objective) equipped with multiple filter sets (Chroma Technology, Rockingham, VT). Excitation light was delivered by a 175-watt Xenon lamp. Images were obtained and analyzed using C-Imaging software program (Compix, Sewickley, PA).

RESULTS

Selection of Appropriate Expression System—Electrophysiological studies of Ca\(^{2+}\) channels are traditionally based on the use of HEK293 cells. However, several independent careful evaluations have shown that these cells contain endogenous Ca\(^{2+}\) channel subunits and exhibit \( I_{\text{Ca}} \) at a level of 1–3 pA/PF (25, 26). Thus, HEK293 cells can be used safely for the functional analysis of recombinant Ca\(^{2+}\) channels only when the amplitude of the current is large enough to ignore the contribution of the endogenous channels. To avoid this problem, in this study we used COS1 cells because they lack endogenous Ca\(_{1.2}\) subunits (27): (a) Western blot analysis with anti-\( \alpha_{1C} \) Ab revealed no detectable endogenous \( \alpha_{1C} \) in non-transfected COS1 cells (Fig. 2A, lane 1), and (b) no Ca\(^{2+}\) channel activity was observed in COS1 cells expressing recombinant \( \alpha_{1C} \) and \( \alpha_{2, \delta} \) subunits (Fig. 2B) in contrast to cells that were co-transfected with \( \beta_{2d} \), \( \beta_{2\delta} \), or \( \beta_{\text{CED}} \) (see below). This experiment unambiguously shows that Ca\(_{1.2}\) calcium channels are silent in the absence of Ca\(_{2}\) and that activity of the Ca\(^{2+}\) channel demonstrated in HEK293 cells in the absence of exogenous Ca\(_{2}\) subunits may be due to endogenous channels. The absence of the functional Ca\(_{2}\) subunits in COS1 cells that follows from the data in Fig. 2B was further confirmed by co-IP analysis with recombinant FLAG\(_N\)-\( \alpha_{1C}/\alpha_{2, \delta} \) that revealed a lack of detectable endogenous monkey \( \beta_{1}, \beta_{2} \), \( \beta_{3} \), and \( \beta_{4} \) subunits in COS1 cells (28). Kinetics parameters and voltage dependence of activation and inactivation of \( I_{\text{Ca}} \) and \( I_{\text{Ba}} \) through the \( \alpha_{1C, \text{77}}/\alpha_{2, \delta}/\beta_{1a} \) channel measured in COS1 cells were consistent with data obtained in other expression systems (15). An important advantage of COS1 cells is their relatively slow division rate that allows for better control over efficiency of expression and assembly of the Ca\(_{1.2}\) channel subunits of different size. However, HEK293 cells were more appropriate for co-IP-Western blot analysis of the recombinant tagged channel proteins in our study because they provide higher efficiency of expression, whereas endogenous subunits were undetectable with streptavidin and Abs to FLAG and Venus (ECFP) tags by Western blot analysis and fluorescence microscopy.

Localization of \( \beta_{\text{CED}} \) by Deletion Analysis of the \( \beta_{2\delta} \) Subunit—To locate \( \beta_{\text{CED}} \), we constructed the following \( \beta_{2\delta} \) fragments: 1–82, 83–164, 83–123, and 124–164 (Fig. 3, A–E, panels a). To ease IP and detection, \( \beta_{2\delta} \) fragments were tagged at the N termini with the monomeric mVenus protein (29) or ECFP. As with other Ca\(_{2}\) subunits (e.g. \( \beta_{1} \), \( \beta_{2} \), \( \beta_{3} \), and \( \beta_{4} \) (19)), fusion of ECFP and mVenus to the N termini of the \( \beta_{2\delta} \) fragments did not markedly change electrophysiological properties of the expressed channels (e.g. \( \beta_{2}(124–164) \) in Fig. 2C). The ECFP\(_N\)-labeled \( \beta_{2\delta} \) fragments were co-expressed with the \( \alpha_{1C, \text{77}} \) and \( \alpha_{2, \delta} \) subunits in COS1 cells. The relative tendency of the ECFP\(_N\)-\( \beta_{2\delta} \) deletion mutants to accumulate in PM can be seen in close juxtaposition from distribution of ECFP fluorescence in the expressing cells (Fig. 3, A–E, panels b and c). Ability of the \( \beta_{2\delta} \) deletion mutants to support \( I_{\text{Ca}} \) was assayed by patch clamp (Fig. 3, A–E, panels d). Binding to \( \alpha_{1C} \) was assayed by co-IP of the mVenus\(_N\)-labeled \( \beta_{2\delta} \) fragments with FLAG\(_N\)-\( \alpha_{1C, \text{77}}/\alpha_{2, \delta} \) (Fig. 3F).

Confirming previous observations (19), when co-expressed with \( \alpha_{1C, \text{77}} \) and \( \alpha_{2, \delta} \) subunits, \( \beta_{2\delta} \) was appreciably accumulated in PM (Fig. 3A, panels b and c), stimulated inward \( I_{\text{Ca}} \) with an average maximal amplitude of 80 ± 15 pA (n = 45; Fig. 3A, panel d), and co-immunoprecipitated with \( \alpha_{1C} \) subunit (Fig. 3F, lane 1). Sequential deletion of the \( \beta_{2\delta} \) subunit (Fig. 3, B–E) revealed that calcium channel activity is associated with the distal quarter of the \( \beta_{2\delta} \) sequence. Only the \( \beta_{2\delta} \) fragments containing the distal C-terminal regions 83–164 (Fig. 3C) and 124–164 (Fig. 3D) induced the current when co-expressed with
**Ca_β_2 Subunit C-terminal Determinant**

α_{t,77}/α_2δ (panels d) and directly interacted with α_{t,77} as evident from the marked accumulation in PM (panels b and c) and Western blot analysis of co-IP with microsomal α_{t,77} (Fig. 3F, lanes 3 and 5). Taken together, results of this analysis show that the C-terminal sequence of 41 amino acids of the Ca_β_2 subunit (β_2CED) represents a previously unknown determinant that may have a role in calcium channel modulation. Amino acid alignment revealed (Fig. 4) that β_2CED is conserved in Ca_β_2 and shares only a subtle homology with the other Ca_β subunits.

**Electrophysiological Properties of the β_2CED-supported Channel**—In COS1 cells expressing EYFP_N-α_{t,77} and α_2δ subunits, the average maximal amplitude of I_Ca decreased from 647 ± 34 pA (n = 48) with β_2δ to 120 ± 25 pA (n = 48) with β_2CED. Fig. 5A shows a family of representative traces of I_Ca evoked by a stepwise depolarization in the range of −20 to +60 mV applied from V_h = −90 mV. The currents were almost completely inhibited by the specific L-type calcium channel blocker (+)PN200-110 (Fig. 5B, traces a). An interesting feature of these currents is the presence of a large slow component of inactivation that is unusual for the Ca^{2+}-conducting Ca_1.2 channels. Analysis of the steady-state inactivation curve (Fig. 5C) showed that at the end of a 1-s conditioning pulse 14.5 ± 1.7% (n = 12) of the peak I_Ca remained non-inactivated. Analysis of −50 investigating cells revealed that I_Ca evoked by V_h between −20 and 0 mV are better fitted with two exponentials and showed a prominent fast component of inactivation (see Table 1). The latter property can be better appreciated from the exemplar I_Ca traces (recorded at −20 and −10 mV) in Fig. 5B that have larger amplitude than the representative currents in Fig. 5A. However, at V_h ≥ 10 mV, the decay of I_Ca was better fitted by a single exponential. The large sustained current (I_{s,s}) (Table 1) is characteristic for all shown voltages and may be indicative of the inhibited slow inactivation (6, 24).

Co-plotting of I-V and τ-V curves (Fig. 5D) showed that when β_2δ in the channel (Table 1) was replaced with β_2CED, inactivation of I_Ca became slower on stronger depolarization and did not depend on the size of the current. The corresponding lack of U-shaped τ-V dependence is evidence that β_2CED does not support CDI (30). To further characterize modulation of inactivation of the Ca_1.2 channel by β_2CED, we tested the effect of replacement of Ca^{2+} for Ba^{2+} in the bath medium on kinetics of the current decay. When Ba^{2+} is the charge carrier, Ca_1.2 channels inactivate by a voltage-dependent mechanism (2). The I-V relationship for I_{ba} (V_{0.5,act} = 41.3 ± 4.3, n = 31, Fig. 5E) was shifted to more positive potentials as compared with I_Ca (V_{0.5,act} = 19.8 ± 1.8, n = 48, Fig. 5D). A ~10-mV positive shift of the steady-state inactivation curve was also observed on replacement of Ca^{2+} (V_{0.5,act} = 13.9 ± 1.1) for Ba^{2+} (V_{0.5,act} = 24.2 ± 3.3) in the bath medium (Fig. 5C), whereas the voltage dependence of availability of the β_2CED-modulated channel was increased to 50.6 ± 2.9% (n = 24) with Ba^{2+} as the charge carrier. These data suggest that the inactivating fraction of channels is reduced in Ba^{2+} because of increased voltage dependence of availability of the β_2CED channel.
The U-shaped dependence of inactivation between the 
whereas those for $I_{Ba}$ was almost flat (Fig. 5A). Confirming the result of the $\tau$-$V$ analysis (Fig. 5D), no U-shaped dependence of $r_{so}$ on $V_t$ was observed for $I_{Ca}$, whereas those for $I_{Ba}$ was almost flat (Fig. 5F). A sharp difference between $r_{so}$ values for $I_{Ba}$ and $I_{Ca}$ at lower voltages reflects a switch from an apparent biexponential voltage-dependent inactivation at $-20$ to $0$ mV to a predominantly single-component mechanism at $V_t \geq 10$ mV (see above). One could argue that this result may be due to a specific level of intracellular Ca$^{2+}$ buffering in our experiment. However, CDI in Ca$_{1.2}$ calcium channels exhibits low sensitivity to intracellular Ca$^{2+}$ buffers (32, 33). The possibility that $\beta_2$CED evokes CDI only in the narrow voltage range of $-20$ to $0$ mV is doubtful because of $\beta_2$CED- and $\beta_2$Ba-modulated channels by superimposing $I_{Ca}$ and $I_{Ba}$ traces near the maximum of $I$-$V$ curves. At $V_t$ between $+20$ and $+40$ mV, where the currents are larger, the $\beta_2$Ba-modulated $I_{Ca}$ inactivated notably faster than $I_{Ba}$ (Fig. 6A) due to CDI. In contrast, we observed a matching decay of $I_{Ca}$ and $I_{Ba}$ for the $\beta_2$CED-modulated channel (Fig. 6B) confirming the lack of CDI. We next compared decays of $I_{Ca}$ and $I_{Ba}$ sampled near the maximum of $I$-$V$ curves (Fig. 5F) at $V_t = 0$ mV, where the CDI effect is negligible (Fig. 6C) due to the lack of CDI and explains the slow kinetics of $I_{Ca}$ decay in the $\beta_2$CED channel by the lack of the negative feedback regulation of inactivation by the permeating Ca$^{2+}$ ions.

Overlaying of $I_{Ca}$ and $I_{Ba}$ traces, scaled to the same amplitude, is a common approach to estimate the contribution of CDI and voltage-dependent mechanisms in inactivation of the channel. In Fig. 6 we compared inactivation properties of the $\beta_2$CED channel, tested for CDI by calculating the $f$ factor (31), which is the difference between the $r_{so}$ values, or fractions of $I_{Ca}$ and $I_{Ba}$ remaining at the end of a 50-ms depolarization. The 50-ms window was selected to accurately account for the relatively fast decay of $I_{Ca}$ in the range of $-20$ to $+10$ mV (Fig. 5A). Confirming the result of the $\tau$-$V$ analysis (Fig. 5D), no U-shaped dependence of $r_{so}$ on $V_t$ was observed for $I_{Ca}$, whereas those for $I_{Ba}$ was almost flat (Fig. 5F). A sharp difference between $r_{so}$ values for $I_{Ba}$ and $I_{Ca}$ at lower voltages reflects a switch from an apparent biexponential voltage-dependent inactivation at $-20$ to $0$ mV to a predominantly single-component mechanism at $V_t \geq 10$ mV (see above). One could argue that this result may be due to a specific level of intracellular Ca$^{2+}$ buffering in our experiment. However, CDI in Ca$_{1.2}$ calcium channels exhibits low sensitivity to intracellular Ca$^{2+}$ buffers (32, 33). The possibility that $\beta_2$CED evokes CDI only in the narrow voltage range of $-20$ to $0$ mV is doubtful because of $\beta_2$CED- and $\beta_2$Ba-modulated channels by superimposing $I_{Ca}$ and $I_{Ba}$ traces near the maximum of $I$-$V$ curves. At $V_t$ between $+20$ and $+40$ mV, where the currents are larger, the $\beta_2$Ba-modulated $I_{Ca}$ inactivated notably faster than $I_{Ba}$ (Fig. 6A) due to CDI. In contrast, we observed a matching decay of $I_{Ca}$ and $I_{Ba}$ for the $\beta_2$CED-modulated channel (Fig. 6B) confirming the lack of CDI. We next compared decays of $I_{Ca}$ and $I_{Ba}$ sampled near the maximum of $I$-$V$ curves. Because of CDI, the $\beta_2$Ba-modulated $I_{Ca}$ inactivated appreciably faster than with $\beta_2$CED (Fig. 6C). However, $I_{Ba}$ through both $\beta_2$CED- and $\beta_2$Ba-modulated channels recorded at the same test voltages showed a very similar decay (Fig. 6D) indicating striking similarity of the voltage-dependent inactivation of the channels. Thus, the Ca$^{2+}$/Ba$^{2+}$ test confirmed lack of CDI in the $\beta_2$CED-modulated channel. Lack of CDI is an unusual property that was not previously observed in Ca$_{1.2}$ channels with native pore-forming $\alpha_{1C}$ subunits.
Ca$_{a_1b_2}$ Subunit C-terminal Determinant

Effect of $\beta_2$CED Deletion from $\beta_2$ on the Ca$_{a_12}$ Calcium Channel—To better understand the functional impact of $\beta_2$CED on Ca$_{a_1b_2}$ subunit modulation of Ca$_{a_12}$ channels, the distal 41-amino acid sequence (identical to those of $\beta_2$CED(124–164)) was genetically deleted from $\beta_2$. Modulation of the Ca$_{a_12}$ channel by the obtained deletion mutant $\beta_2$CED was compared with those of $\beta_2$ (Fig. 7). Similar to other Ca$_{a_1b}$ subunits, $\beta_2$ facilitated large $I_{Ca}$ through the Ca$_{a_12}$ channel. Distinct features of $I_{Ca}$ through the $\beta_2$ channel include: 1) a relatively large sustained component of the current that comprised ~35% of the peak current at the end of a 600-ms depolarization pulse (Fig. 7A), and 2) a prominent shift of the maximum $I$-$V$ curve from a typical value of +20 to +30 mV (15) to +40 mV (Fig. 7C, closed circles). As expected, $\beta_2$ supported CDI and showed a U-shaped $\tau$-$V$ dependence of $I_{Ca}$ peaked near the maximum of $I$-$V$ curve (Fig. 7C, open circles).

The $\beta_2$CED-modulated channel generated large inward $I_{Ca}$ (average maximal amplitude 466 ± 160 pA, $n$ = 12) in response to depolarization in a characteristic range of membrane potentials (Fig. 7B). Similar to the $\beta_2$-modulated channel, decay of $I_{Ca}$ at all tested potentials was better fitted by a single exponential. The $\tau$-$V$ relation had a distinct U-shape indicating that deletion of the $\beta_2$CED from the $\beta_2$ subunit did not compromise CDI (Fig. 7D). The maximal inactivation rate of $I_{Ca}$ through the $\beta_2$CED-modulated channel was faster ($\tau$ = 43 ± 5 ms at +10 mV) than that modulated by $\beta_2$ ($\tau$ = 59 ± 6 ms at +20 mV). The voltage dependence of activation ($V_{0.5,act}$) and inactivation ($V_{0.5,in}$) of the $\beta_2$CED-modulated channel were shifted by ~12 (Fig. 7, C and D) and 5 mV (Fig. 7, E and F) to more negative voltages, respectively, suggesting that deletion of $\beta_2$CED endows the channel a higher voltage sensitivity (for statistics, see figure legend). Taken together, these results and data in Fig. 5 point to a synergy between MAGUK and $\beta_2$, but they may act independently as modulators of the Ca$_{a_12}$ channel.

At present, the prevailing view suggests that AID is a constitutive binding site for all known Ca$_{a_1b}$ subunits. To further test whether deletion of $\beta_2$CED from the $\beta_2$ subunit interferes with binding to AID, we used the I-I12 peptide (34). To ease IP and detection, I-I12 was tagged at the N terminus with the monomeric mVenus protein (29). The mVenusN-labeled I-I12 was co-expressed with FLAGN-tagged $\beta_2$ (Fig. 7G, lane 1) or $\beta_2$CED (lane 2) in HEK293 cells. Consistent with the results of electrophysiological experiments (Fig. 7, A–F), Western blot analysis of co-IP showed that deletion of $\beta_2$CED from $\beta_2$ did not compromise binding of $\beta_2$CED to I-I12 as compared with $\beta_2$ (Fig. 7G).

$\beta_2$CED Supports Ca$_{a_12}$ Channels in the Absence of AID—When mVenussC-$\beta_2$CED and BiotinN-I-I12 were co-expressed in HEK293 cells (Fig. 8A), $\beta_2$CED was identified (lane 1) on Western blot by monoclonal anti-LC Ab in both the immunoprecipitated (top left panel) and input (top right panel) fractions. However, I-I12 was detected on the blot by streptavidin only in the input fraction (bottom panel). Thus, co-IP analysis indicates that $\beta_2$CED does not bind to AID. These data suggest that $\beta_2$CED exerts its modulation of the Ca$_{a_12}$ channel through interaction with a site(s) other than AID.

To confirm this conclusion, we abolished binding of the $\beta$ subunit MAGUK domain to $\alpha_{1C}$ by the combined substitution with alanine at four key positions (Asp$^{433}$, Gly$^{436}$, Tyr$^{437}$, and Trp$^{440}$) of the $\alpha_{1C}$ I-II linker (10, 12, 35). Co-IP assay (Fig. 8B) confirmed that binding of $\beta_2$ to I-I12 (lane 1) was abolished by the AID mutation independently on the presence (lane 2) or absence (lane 3) of $\beta_2$CED. The AID mutation was then incorporated into the $\alpha_{1C727}$ subunit, and the resulting mutant $\alpha_{1C727}$AIDM was co-expressed in COS1 cells with $\alpha_{1C}$ and $\beta_2$ (Fig. 8C), $\beta_2$CED (Fig. 8D), $\beta_2$ (Fig. 8E), or $\beta_2$CED (Fig. 8F).
Electrophysiological experiments showed that, despite the inhibited binding between MAGUK and AID (Fig. 8B), β2d facilitated I_Ca through the mutated α1C,77AIDM channel that showed little, if any, inactivation (Fig. 8C). As it is shown in Fig. 2B, no current could be detected in COS1 cells expressing α1C/δ in the absence of Ca_β. The AID mutation did not inhibit conductance completely, but reduced the amplitude of the maximal I_Ca induced by β2d to 131 ± 25 pA (n = 5) suggesting that the channel activation outside of AID by the full-size Ca_β does occur, but is less effective than that with the participation of intact AID. The same conclusion was obtained with β2dACED, β2dA′g and β2dC. When β2d was substituted for β2dACED (Fig. 8D), we observed a functionally active channel that exhibited a slowly inactivating I_Ca with an average maximum amplitude of 150 ± 24 pA (n = 6). Similar to β2dA′ (Fig. 8E), inactivation of a large fraction of I_Ca was inhibited by substitution of β2d for β2dC (Fig. 8F), whereas the average amplitude of the peak I_Ca through the α1C,77AIDM channel was smaller (40 ± 15 pA, n = 3). No appreciable modulation of the α1C,77AIDM channel (i.e. zero I_Ca) was observed in the absence of Ca_β (Fig. 8G). Thus, inhibition of the MAGUK domain binding to AID did not abolish the sensitivity of the channel to β2dC. These data confirm that essential regulatory properties of Ca_β are AID-independent (17) and show that β2dC can serve as a weak I-II linker-independent activator of the Ca_1.2 channel even when AID is mutated causing large conformational changes in the I-II loop.

**Analysis of β2dC Interaction with the LA/IQ Region of α1C—**

A meaningful characterization of the β2dC modulation of the channel requires identification of its functional target. A recent report (36) demonstrated that the N-terminal domain of MAGUK in Ca_β binds to the α1C subunit C-terminal region (amino acids 1571–1636 in α1C,77) that is involved in Ca_2+-mediated CDI regulation and includes LA and IQ loci of interaction with apo-CaM and Ca^2+/-CaM, respectively (37) (Fig. 1). Because β2dC does not support CDI, we tested β2d (containing β2dC) and β2dACED (lacking β2dC) for binding to LA/IQ. Co-IP analysis showed (Fig. 9) that LA/IQ binds β2d and β2dACED independently on the presence of I-IIAID; moreover, β2d and β2dACED bound to LA/IQ do not form triple complexes with I-IIAID.

We then tested β2dC for binding to LA/IQ. The FLAGN-tagged LA/IQ domain was co-expressed with mVenusN-β2dC (Fig. 10) in the presence (lanes 1 and 2) or absence of ECFP, CaM (lanes 3 and 4). To assess for Ca^2+ dependence of binding, cells were permeabilized for external Ca^2+ before co-IP by incubating with ionophore ionomycin (5 μM) in the bath medium containing 2 mM EGTA (lanes 1 and 3) or 2 mM Ca^2+ (lanes 2 and 4). Co-IP analysis confirmed that β2dC binds to LA/IQ independently on Ca^2+ or co-expressed CaM.

Is LA/IQ the only region necessary for β2dC action? To answer this question, we deleted LA/IQ from the α1C,77 subunit and co-expressed the resulting α1C,77AIDM mutant with α1C δ and β2d or β2dC (Fig. 11A). β2d modulated the channel via
\[ \text{Ca}_{\beta_2} \text{ Subunit C-terminal Determinant} \]

\[
\begin{array}{c|c|c}
\text{IP anti-FLAG} & \text{Input} & \text{kDa} \\
\hline
\text{ECFP-N-CaM} & \text{ct 1 2 3 4} & 49 \\
\text{mVenUS-N-\beta_2CED} & \text{ct 1 2 3 4} & 38 \\
\text{mVenUS-N-\beta_2CED} & \text{anti-FLAG} & 6 \\
\text{FLAG-N-LA/IQ} & \text{anti-LC} & \\
\text{mVenUS-N-\beta_2CED (0.5)} & \text{anti-LC} & \\
\text{mVenUS-N-\beta_2CED (0.5)} & \text{anti-FLAG} & \\
\text{mVenUS-N-\beta_2CED} & \text{anti-LC} & \\
\text{Ca}^{2+} (\mu \text{M}) & 0 & \\
\end{array}
\]

\text{FIGURE 10.} \text{\beta_2CED binds to LA/IQ domain independently on Ca}^{2+} \text{ and CaM. Flag-N-LA/IQ was co-expressed with mVenus (control, lane c) or mVenUS-N-\beta_2CED (lanes 1–4) in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of ECFP-N-CaM. Before co-IP, cells were incubated for 5 min in 10 mM HEPES, 135 mM NaCl (pH 7.4), containing 5 \mu M of ionomycin and either 2 mM EGTA or 2 mM CaCl_2 (lanes 2 and 4). Proteins were co-IP with anti-FLAG Ab and resolved by SDS-PAGE. Upper panels, identification of CaM and \beta_2CED on Western blot by monoclonal anti-LC Ab (lanes 1–4). Lower panels, identification of LA/IQ by anti-FLAG Ab. The amount of plasmid DNA (\mu g) per transfection reaction is shown in parentheses.}

\text{A} \quad \alpha_{1C,77ALK}/\alpha_2\delta/\beta_2d \\
\text{a} \quad \alpha_{1C,77ALK}/\alpha_2\delta/\beta_2CED \\
\text{b} \quad \alpha_{1C,77ALK}/\alpha_2\delta/\beta_2CED \quad 100 \text{ pA} \\
\text{200 ms} \\
\text{B} \quad \alpha_{1C,77LM}/\alpha_2\delta \\
\text{a} \quad \alpha_{1C,77LM}/\alpha_2\delta/\beta_2CED \\
\text{b} \quad \alpha_{1C,77LM}/\alpha_2\delta/\beta_2CED \quad 100 \text{ pA} \\
\text{200 ms} \\
\text{C} \quad \alpha_{1C,77KL}/\alpha_2\delta \\
\text{a} \quad \alpha_{1C,77KL}/\alpha_2\delta/\beta_2CED \\
\text{b} \quad \alpha_{1C,77KL}/\alpha_2\delta/\beta_2CED \quad 100 \text{ pA} \\
\text{200 ms} \\
\text{D} \quad \alpha_{1C,77KL}/\alpha_2\delta/\beta_2d \\
\text{a} \quad \alpha_{1C,77KL}/\alpha_2\delta/\beta_2d \\
\text{b} \quad \alpha_{1C,77KL}/\alpha_2\delta/\beta_2d \quad 200 \text{ ms} \\
\text{E} \quad \alpha_{1C,77AIMD/ALK}/\alpha_2\delta/\beta_2d \\
\text{200 ms} \\
\text{100 pA} \\

\text{MAGUK/AID interaction and induced I}_{Ca} \text{ with an average maximal amplitude } \sim 90 \pm 25 \text{ pA (panel a, } n = 3). \text{ Under the same conditions, } \beta_2CED \text{ no appreciable } I_{Ca} \text{ was observed on cell depolarization (panel b). This result suggests that the LA/IQ region is the only functional target of the } \alpha_{1C,77} \text{ subunit where } \beta_2CED \text{ may exert its action. To test whether LA or IQ determinants of CDI are essential for modulation of the channel by } \beta_2CED, \text{ we examined the effect of } \beta_2CED \text{ on the } \alpha_{1C,77} \text{ mutants lacking IQ (Fig. 11B, } \alpha_{1C,77L}) \text{ or LA (Fig. 11C, } \alpha_{1C,77K}) \text{ determinants defined in Fig. 1. In the absence of } \alpha_{1C,77}, \text{ none of the tested channels showed appreciable } I_{Ca} \text{ in response to } V_h = +20 \text{ mV applied from } V_m = -90 \text{ mV for } 600 \text{ ms (Fig. 11, B and C, panels a). Co-expression of } \beta_2CED \text{ induced } I_{Ca} \text{ only with } \alpha_{1C,77L} \text{ (average maximal amplitude } \sim 208 \pm 28 \text{ pA, } n = 5, \text{ see Fig. 11B, panel b) indicating that it is the LA determinant of CDI that is the functional target of } \beta_2CED \text{ modulation of the channel. Under the same conditions, } \beta_2d \text{ supported } I_{Ca} \text{ through both } \alpha_{1C,77L} \text{ (Fig. 11D, panel a; average maximal amplitude } 231 \pm 13 \text{ pA, } n = 14) \text{ and } \alpha_{1C,77K} \text{ channels (Fig. 11D, panel b; average maximal amplitude } 389 \pm 24 \text{ pA, } n = 8). \text{ However, mutation of AID combined with the deletion of LA/IQ from the } \alpha_{1C} \text{ subunit } \alpha_{1C,77AIMD/ALK} \text{ completely inhibited modulation of the channel by } \beta_2d \text{ (Fig. 11E).}

\text{DISCUSSION}

\text{Our study revealed that modulation of Ca}_{1.2} \text{ channels by large Ca}_{\alpha,\beta_2} \text{ subunits is mediated by inputs from multiple binding sites. There are at least three interactions between } \alpha_{1C} \text{ and Ca}_{\alpha,\beta_2} \text{ subunits (Fig. 12, A–C) that induce activity of the channel not only jointly, but also when any two of the interactions are disrupted by mutations of } \alpha_{1C}. \text{ The } Ca_{1.2} \text{ channel modulation common to all large } Ca_{\alpha,\beta} \text{ subunits is supported by the binding of the central MAGUK domain to the AID site of the } \alpha_{1C} \text{ subunit I-II linker (4) (Fig. 12A). This interaction stabilizes the functional conformation of AID (and, respectively, the I-II linker), as well as provides specific orientation of the rigid core of } Ca_{\alpha,\beta} \text{ important for multiple isoform-specific interactions leading to differential modulation of the channel (15, 17). However, disruption of this interaction by the mutation of AID (Fig. 8B) does not prevent activation of the channel by } Ca_{\alpha,\beta} \text{ that relies on two other } \alpha_{1C} \text{ determinants located in the LA-IQ region (Figs. 5 and 8, C, D, and F). Deletion of these LA/IQ}

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determinants partially inhibited activity of the channel in the presence of $\beta_{2d}$ as can be seen from the smaller $I_{Ca}$ amplitude (Fig. 11A, panel a). A dynamic Ca$^{2+}$-dependent interaction between the N-terminal SH3 region of MAGUK and the IQ domain of the $\alpha_{1C}$ subunit (36) (Fig. 12B) appears to also be common to all large Ca$\beta$ subunits. This interaction alone is sufficient to support $I_{Ca}$ (Fig. 8D).

In this study, we localized the third molecular determinant of the $\alpha_{1C}$ channel modulation, $\beta_{2}$CED (Fig. 12C), which is specific only to $\alpha_{1C}$ (Fig. 4) and resides in the C terminus of $\beta_{2a}$, $\beta_{2b}$, $\beta_{2c}$, $\beta_{2d}$, $\beta_{2f}$, and $\beta_{2g}$ subunits. Thus, $\beta_{2}\text{CED}$ represents a functional element of the $\alpha_{1C}$ modulation that is conserved in primary cardiac Ca$\beta$ subunits. In the case of full size Ca$\beta$ subunits ($\beta_{2d}$–$\beta_{2w}$) (20), $\beta_{2}$CED acts in synergy with other determinants, as seen from the ability of $\beta_{2}$CED and $\beta_{2d}\Delta\text{CED}$ to support $I_{Ca}$ with different properties (Figs. 5 and 7). However, $\beta_{2}$CED may induce activity of the channel independently on MAGUK, i.e. either alone (Fig. 5) or in the context of “short” $\beta_{2}$ subunits, as in the case of the naturally occurring $\beta_{2a}$ subunit (19) (Fig. 8E). Similar to other Ca$\beta$ subunits, $\beta_{2}$CED activates the channels by binding to $\alpha_{1C}$ (Fig. 3F) and targeting to PM (Fig. 3E), but does not support CDI (Fig. 5D). It appears that $\beta_{2}$CED affects voltage gating of the channel. Indeed, similar to $\beta_{2d}$ (Fig. 7C), the $\beta_{2}$CED-modulated channel has the maximum $I$-$V$ curve at + 40 mV (Fig. 5D), whereas deletion of $\beta_{2}$CED from $\beta_{2d}$ shifted the voltage dependence of $I_{Ca}$ by 20 mV to lower potentials (Fig. 7D). We find that $\beta_{2}$CED binds to the LA/IQ region of the $\alpha_{1C}$ subunit C-terminal tail in a Ca$^{2+}$- and CaM-independent manner and needs the LA, but not IQ, motif to activate the channel (Fig. 10). This is the first observation of the Ca$\beta_{2}$ subunit regulation of the Ca$\alpha_{1C}$ calcium channel that does not rely on Ca$\beta$/AID interaction.

Although $\beta_{2}$CED did not bind to AID (Fig. 8A), mutation of AID interfered with the interaction of the channel with $\beta_{2}$CED that was reflected in the smaller amplitude of $I_{Ca}$ (cf. Figs. 5A and 8F). This result points to high sensitivity of all three $\alpha_{1C}$/Ca$\beta_{2}$ interactions (Fig. 12) to the conformation of AID that is probably a key component of mutually dependent determinants of channel regulation (7, 24).

Another important conclusion from our study is that CDI does not depend solely on the $\alpha_{1C}$ subunit. Indeed, co-expression of intact $\alpha_{1C}$ and $\alpha_{1}\delta$ subunits with $\beta$CED generates the channel lacking CDI (Figs. 5 and 6). It is known that CDI is mediated by interactions of CaM with two adjacent sites (LA and IQ) of the $\alpha_{1C}$ subunit C terminus (for review, see Ref. 38). We found that CDI ultimately requires both MAGUK/AID and SH3/IQ interactions (Fig. 12, A and B). Thus the role of Ca$\beta$, AID, and LA/IQ interactions in the ensemble of mutually coordinated determinants of CDI is essential.

The exact number of Ca$\beta$ subunits (of the same or different type) that bind to an individual $\alpha_{1C}$ subunit is unknown. Results in Fig. 9 show that $\beta_{2d}$ is not involved in a simultaneous binding to LK and AID, although this subunit can be engaged in all three types of interactions shown in Fig. 12. Therefore, it is possible that there is more than one Ca$\beta$ subunit interacting with the same $\alpha_{1C}$. On the other hand, if Ca$\beta$ disassociates from the AID of the functional channel (39), then it is possible that modulation of the channel may be mediated by a single Ca$\beta_{2}$ molecule alternating between AID and LA/IQ sites. An additional complexity (36) to this general picture may be brought about by the N-terminal palmitoylation site known to anchor the $\beta_{2a}$ subunit in PM (40).

Taken together, our results provide new insight into potential role(s) of $\beta$CED in modulation of Ca$\alpha_{1C}$ channels. Ca$\beta_{2}$ is a major cardiac $\beta$ subunit and its splice variation is an important correlate of the Ca$\alpha_{1C}$ calcium channel regulation (20, 41). One of the most puzzling questions raised by our study is why Ca$\beta$ subunits contain more than one $\alpha_{1C}$ interaction motif. One possible reason for this complexity may be associated with the role of $\beta$CED in additional tuning of the voltage dependence of the current (20). Indeed, results of electrophysiological measurements (Fig. 7) show that deletion of $\beta_{2}$CED from $\beta_{2d}$ significantly changed kinetics of inactivation of $I_{Ca}$, and shifted the peak of the $I$-$V$ curve by 20 mV toward more negative potentials. Another reason may be that differential, tissue-specific splicing of the Ca$\beta_{2}$ gene (18) may generate subsets of the Ca$\alpha_{1C}$ calcium channel modulated only through $\beta$CED. These channels do not support CDI and generate small, but long-lasting Ca$^{2+}$ currents. It is usually assumed that $I_{Ca}$ is rapidly and fully inactivated, but our results raise the hypothesis that Ca$^{2+}$ signaling in human cardiac cells expressing small Ca$\beta_{2}$ subunits (19) may involve Ca$^{2+}$-insensitive Ca$\alpha_{1C}$ calcium channels in addition to L-type channels regulated by CDI. One possibility is that Ca$^{2+}$ channels that rely on $\beta$CED-dependent, MAGUK-independent modulation in cardiac muscle cells may account
for the prolongation of L-type I_{Ca} and therefore contribute to the balance that controls the shape of the action potential plateau. Whichever role of β_{2}CED is predominant, it may be a new potential pharmacological target.

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