Supporting Information

Fan et al. 10.1073/pnas.1007543107

SI Materials and Methods

Constructs and Cloning. For coimmunoprecipitation experiments in HEK293T cells (gift of Dr. Hiroaki Matsunami, Duke University, Durham, NC), an HA tag was added to the N terminus of full-length Gem cDNAs (GenBank accession number BC022010) from human skeletal muscle with a flexible linker of three glycines in between, and the whole construct was cloned into the pCDNA3.1(−) vector (Invitrogen). A Myc tag was added to the N terminus of full-length rat brain β_3 (GenBank accession number M88751) with three glycines in between, and the whole construct was cloned into the pCDNA3.1(−) vector (Invitrogen). Rabbit brain $Ca_v2.1$ (GenBank accession number 57477) was subcloned into the p3xFLAG-CMV-7.1 vector (Invitrogen).

For electrophysiology experiments in *Xenopus* oocytes, cDNAs encoding various constructs were subcloned into a modified oocyte expression vector pGEMHE. The constructs included wild-type (WT) or mutated rabbit brain $Ca_v2.1$, WT or mutated mouse $Ca_v3.1$ (GenBank accession number AJ012569), rat skeletal muscle $\alpha_2\delta$, WT or mutated rat brain β_3, and WT or mutated human skeletal muscle Gem.

For protein synthesis in *Escherichia coli*, BL21(DE3) bacteria were used for cDNA transformation and proteins expression. Gem(S68-K276) cDNA encoding residues S68 to K276 were subcloned into the pAcycDuet vector (Novagen). β_3_core cDNA encoding residues G16 to G366 was subcloned into the pET-26b vector (Novagen).

Cell Culture and Transfection. HEK293T cells were maintained at 37 °C in a DMEM medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Lipofectamine 2000 (Invitrogen) was used for all transfections.

SDS/PAGE and Western Blot. SDS/PAGE was performed in a Tris-glycine-SDS buffer with 8% or 12% acrylamide gel. Precision Plus Protein All Blue Standards (Bio-Rad) were used as molecular-weight (MW) markers.

For Western blot, after electrophoresis, the protein gel was transferred to the PVDF membrane and processed with the Odyssey Western blot kit (Li-Cor). The monoclonal mouse anti-HA antibody HA.11 (Covance), the monoclonal mouse anti-Myc antibody (Sigma), or the monoclonal mouse anti-FLAG antibody M2 (Sigma) was used as the primary antibody. Alexa Fluor 680 goat anti-mouse IgG (Invitrogen) was used as the secondary antibody. Images were scanned and analyzed with the Odyssey Infrared Imaging System (Li-COR).

Oocyte Preparation and Expression. Ovarian lobes were obtained from adult *Xenopus laevis* (*Xenopus* I) under anesthesia. Stages V–VI oocytes were prepared by treatment with 2.5 mg/mL collagenase A (Boehringer Mannheim) for 1.5–2.5 h under 200 rpm shaking in a solution containing 82.4 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 5 mM hepes (pH 7.6), and then, they were rinsed two times (15 min each) with ND96 solution containing 96 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM hepes, 1.8 mM $CaCl_2$, 100 units/mL penicillin, and 100 μg/mL streptomycin (pH 7.6). Single defoliculated oocytes were individually selected. cRNAs were synthesized in vitro, and varying amounts (0.2–5 ng) were injected into selected oocytes in various combinations. Recordings were performed 3–5 d after injection.

Electrophysiology. All experiments were performed at 22 °C.

For whole-oocyte recordings by two-electrode voltage clamp, electrodes were filled with 3 mM KCl and had a resistance of 0.5–1 MΩ. The bath solution contained (in mM) 10 $BaCl_2$, 5 KCl, 60 tetra-ethyl ammonium hydroxide, 20 NaOH, and 5 hepes (pH 7.4 with HCl). The current was evoked every 6 s by a +20 mV pulse for 40 ms from a holding potential of −80 mV. For experiments with chimeras, the bath solution contained (in mM) 40 $Ba(OH)_2$, 50 NaOH, 2 KCl, 2 $BaCl_2$, and 5 hepes; pH was adjusted to 7.4 using methanesulfonic acid, and the solution was filtered. Voltage steps were 50- or 1,500-ms long and were applied from a holding potential of −100 mV.

For inside-out macropatch recordings from oocytes, the electrode had a diameter of 15–30 μm and a resistance of 0.2–0.5 MΩ when filled with a solution containing (in mM) 45 $BaCl_2$, 80 KCl, and 10 hepes (pH 7.3 with KOH). The bath (i.e., cytoplasmic) solution contained (in mM) 125 KCl, 4 NaCl, 10 hepes, and 10 EGTA (pH 7.3 with KOH); 0.3 μM PIP_2 and 3 mM Mg-ATP were added freshly to the bath solution to attenuate rundown. Control and test solutions were fed by a pressurized system through separate tubes to a manifold attached to a single outlet tube and were switched on/off individually. After obtaining an inside-out patch, the recording pipette was inserted into the perfusion tube to achieve a rapid and complete exchange of solution. Macroscopic currents were evoked from a holding potential at −80 mV by 10-ms depolarizations ranging from −30 mV to +90 mV in 10-mV increments at a 1-s interval. Currents were sampled at 10 kHz and filtered at 2.5 kHz.

To obtain the β-less channels described in Fig. 2, a fast perfusion speed (~1.5 mL/min) was used. Macroscopic currents were evoked from a holding potential at −80 mV by 10-ms depolarizations ranging from −30 mV to +90 mV in 10-mV increments at a 1-s interval. To obtain the activation curve, macroscopic currents were evoked by 20-ms depolarizations ranging from −40 mV to +100 mV in 10-mV increments at a 6-s interval. Tail currents were recorded by repolarization to −40 mV, regardless of the preceding test pulse, normalized by that after the depolarization to +100 mV, and plotted against the test potentials. Currents were sampled at 10 kHz and filtered at 2.5 kHz.

All data were analyzed with Clampfit and were represented as mean ± SD (number of observations). Significance was determined using two-tailed Student *t* test.

Coimmunoprecipitation. About 30–48 h later after transfection, cells were collected in the PBS buffer (pH 7.2; Gibco) at 4 °C. Cell lystates were obtained by adding 1% (V/V) Triton-x100 (Sigma) and 1/50 (V/V) Protease Inhibitor Mixture (Sigma) and rotating at 4 °C for 1 h. Supernatant was obtained by centrifuging at 11,000 × *g* for 20 min and was mixed with monoclonal anti-HA antibody (HA.11)-coated beads (Covance). After incubation at 4 °C for overnight, the beads were spun down and washed three times with a solution containing 1× PBS with a total NaCl concentration of 250 mM and 1/50 (V/V) Protease Inhibitor Mixture (Sigma). The bound proteins were then eluted by adding 0.4 mg/mL HA peptide (Genscript), boiled with an SDS loading buffer for 10 min, and analyzed by SDS/PAGE and Western blot.

Protein Synthesis in *E. coli*. Transformed BL21(DE3) bacteria were cultured at 37 °C until OD_{600} reached 0.6 and then, were induced at room temperature by 1 mM isopropyl β-D-1-thiogalactopyranoside overnight. Cells were collected at 2,500 × *g* for 15 min and resuspended in a lysis solution containing 50 mM Tris-HCl, 250 mM NaCl, 2.5% glycerol, and 7 mM β-mercaptoethanol (pH

7.8). Resuspended bacteria were sonicated with a Branson digital sonifier followed by centrifugation at 11,000 × *g* for 40 min. The supernatant was collected and incubated with Ni-NTA His•Bind beads (Novagen) in the presence of 20 mM imidazole at 4 °C for 1 h. The beads were centrifuged at 650 × *g* for 1 min, collected, and washed with 20 volumes of the above lysis solution containing 20 mM imidazole and 3 volumes containing 50 mM imidazole. Proteins were eluted from the beads with 200 mM imidazole in the lysis solution. Gem(S68-K276) protein was further purified with a Superdex 75 gel-filtration column (Pharmacia) in a solution containing 20 mM hepes and 500 mM NaCl (pH 7.8 with NaOH). β_3_core protein was further purified with a Superdex 75 gel-filtration column in a solution containing 20 mM hepes and 250 mM NaCl (Ph 7.2 with NaOH).

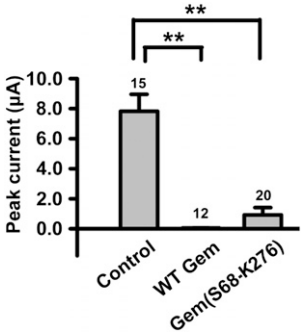

Fig. S1. Inhibition of whole-oocyte P/Q-type Ca^{2+} channel currents by constitutively expressed WT Gem and Gem(S68-K276).

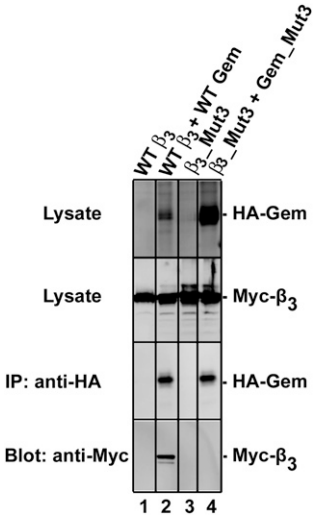

Fig. S2. This figure is the same as Fig. 3*D* but shows larger portions of the gels.

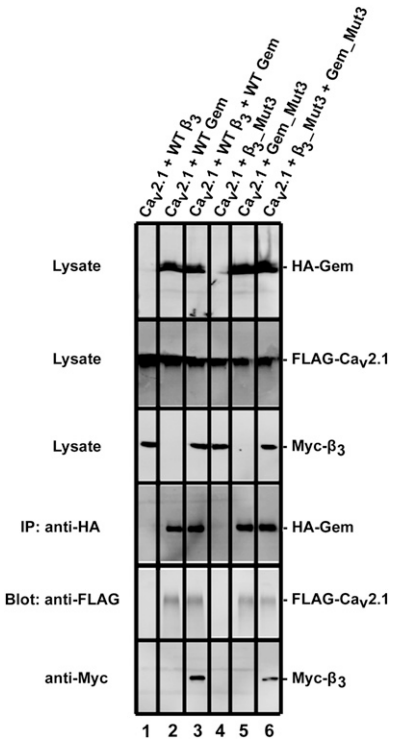

Fig. S3. This figure is the same as Fig. 4*A* but shows larger portions of the gels.

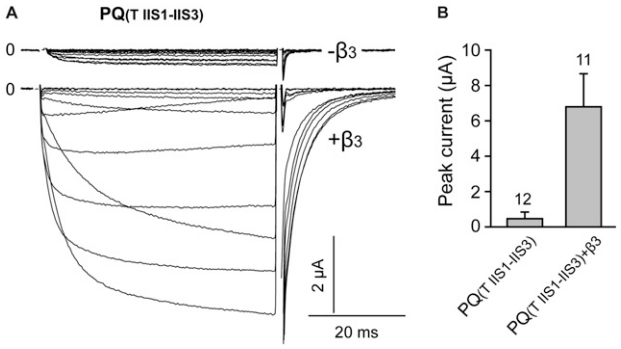

Fig. S4. The β_3 subunit enhances the current produced by $PQ_{(T\ IIS1—IIS3)}$. (*A*) Representative family of currents recorded by two-electrode voltage clamp from oocytes expressing $PQ_{(T\ IIS1—IIS3)}$, without or with β_3. (*B*) Comparison of peak currents in oocytes expressing $PQ_{(T\ IIS1—IIS3)}$, without or with β_3.

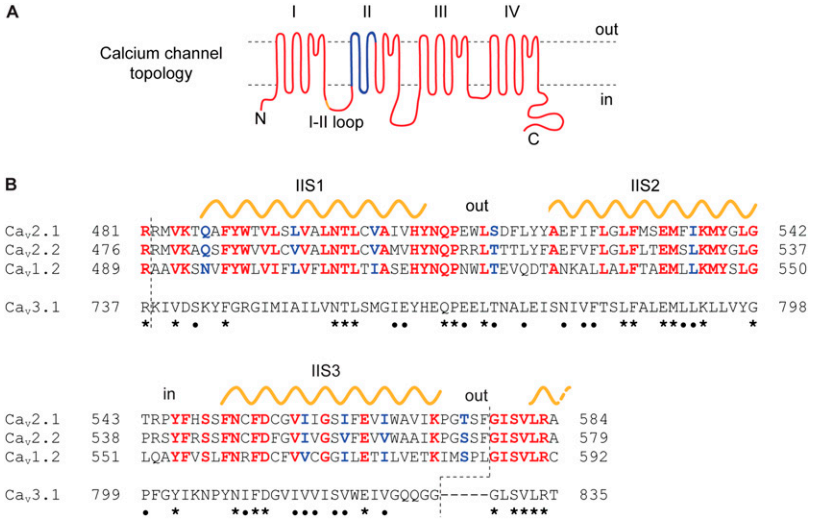

Fig. S5. (*A*) Transmembrane topology of the α_1 subunit of voltage gated Ca^{2+} channels. Blue marks the region exchanged in the P/Q- and T-type channel IIS1–IIS3 chimeras, and yellow in the I–II loop marks the AID. (*B*) Amino acid sequence alignment of the IIS1–IIS3 region of the indicated VGCC α_1 subunits. $Ca_v2.1$, $Ca_v2.2$, $Ca_v1.2$, and $Ca_v3.1$ encode P/Q-, N-, L-, and T-type Ca^{2+} channels, respectively (GenBank accession numbers are X57477, M94172, L04569, and AJ012569). Transmembrane segments IIS1, IIS2, and IIS3 are marked by the helices; extracellular and intracellular loops are indicated by out and in. Red and blue indicate identical and similar residues, respectively, among $Ca_v2.1$, $Ca_v2.2$, and $Ca_v1.2$. Asterisks and dashes indicate residues in $Ca_v3.1$ that are identical to those in all or one of the other three subunits, respectively. Dotted vertical lines mark the start and end points of IIS1–IIS3 chimeras. Amino acid residue numbers are given on both sides of the sequences.