N Terminus Is Key to the Dominant Negative Suppression of Ca\textsubscript{V}2 Calcium Channels

WARDING FOR EPISODIC ATAXIA TYPE 2

Expression of the calcium channels Ca\textsubscript{V}2.1 and Ca\textsubscript{V}2.2 is markedly suppressed by co-expression with truncated constructs containing Domain I. This is the basis for the phenomenon of dominant negative suppression observed for many of the episodic ataxia type 2 mutations in Ca\textsubscript{V}2.1 that predict truncated channels. The process of dominant negative suppression has been shown previously to stem from interaction between the full-length and truncated channels and to result in downstream consequences of the unfolded protein response and endoplasmic reticulum-associated protein degradation. We have now identified the specific domain that triggers this effect. For both Ca\textsubscript{V}2.1 and Ca\textsubscript{V}2.2, the minimum construct producing suppression was the cytoplasmic N terminus. Suppression was enhanced by tethering the N terminus to the membrane with a CAAX motif. The 11-amino acid motif (including Arg\textsuperscript{52} and Arg\textsuperscript{54}) within the N terminus, which we have previously shown to be required for G protein modulation, is also essential for dominant negative suppression. Suppression is prevented by addition of an N-terminal tag (XFP) to the full-length and truncated constructs. We further show that suppression of Ca\textsubscript{V}2.2 currents by the N terminus-CAAX construct is accompanied by a reduction in Ca\textsubscript{V}2.2 protein level, and this is also prevented by mutation of Arg\textsuperscript{52} and Arg\textsuperscript{54} to Ala in the truncated construct. Taken together, our evidence indicates that both the extreme N terminus and the Arg\textsuperscript{52}, Arg\textsuperscript{54} motif are involved in the processes underlying dominant negative suppression.

Voltage-gated calcium (Ca\textsubscript{V}) channels are required for a number of essential physiological processes; in particular, they are essential for many neuronal functions, including neurotransmitter release (for review, see Ref. 1). They are hetero-meric complexes consisting of the pore-forming Ca\textsubscript{V}\alpha\textsubscript{1} subunit together (except in the case of the Ca\textsubscript{V}3 channels) with an accessory \beta and \alpha\textsubscript{\textgamma}\delta subunit. The Ca\textsubscript{V}\alpha\textsubscript{1} subunit consists of four homologous domains (I–IV), each consisting of six transmembrane (TM) segments (see Fig. 1A). The domains are linked by intracellular loops and have intracellular N and C termini. Ten mammalian \alpha\textsubscript{1} subunit genes have been cloned and divided into three subfamilies Ca\textsubscript{V}1–3 (2).

Mutations of calcium channel \alpha\textsubscript{1} subunits can contribute to a number of pathological states (3). In particular, mutations in the CACANIA gene encoding Ca\textsubscript{V}2.1 result in familial hemiplegic migraine and episodic ataxia type 2 (4). Many of the episodic ataxia type 2 mutations in Ca\textsubscript{V}2.1 predict truncated forms of this channel, although missense mutations are also found (4–7). This disease is dominant, and thus there is one wild-type (WT) allele and one mutant allele, both of which are likely to be expressed, although nonsense-mediated decay would reduce the expression of some mutant alleles (8). In many cases, the mutant channels, as well as either being nonfunctional or having reduced functionality, are dominant negative, in that they also suppress the function of the WT channel (9–11).

In our initial study on truncated Ca\textsubscript{V}\alpha\textsubscript{1} subunits, we found that truncated constructs containing Domain I suppressed Ca\textsubscript{V}2.2 currents and reduced the level of full-length Ca\textsubscript{V}2.2 protein (12). We then showed that for both Ca\textsubscript{V}2.2 and Ca\textsubscript{V}2.1, this suppression required interaction between the full-length and the mutant construct (9). In this study, we also examined the effect of a two-domain construct predicted by an episodic ataxia type 2 mutation (9). We and others have also identified previously that the suppressive mechanism involves a reduction in protein synthesis resulting from the unfolded protein response (9) and an acceleration of proteasome-mediated decay (10).

Here, we have dissected the determinants required for suppression, which has increased our understanding of the mechanisms involved in the pathophysiology of episodic ataxia type 2. We find that the interaction between a truncated construct and a related full-length channel, identified previously (9), requires the presence of the N terminus on either or both of the full-length or the truncated channels. We also show that the N terminus of Ca\textsubscript{V}2.2 or Ca\textsubscript{V}2.1 alone is sufficient to suppress expression of the full-length channel. Suppression can be prevented by incorporation of a bulky tag on the N terminus or by removal of part of the N terminus. We further identify the
motifs within the N terminus that are essential for suppression to occur and show that suppression can also be induced of endogenous channels in neurons.

**EXPERIMENTAL PROCEDURES**

**Full-length, Mutant, and Truncated CaV Constructs**—The following cDNAs were used: CaV2.2 (GenBank Accession number D14157), α2δ-1 (GenBank Accession number M86621), αβ-2 (13), β1b (14), CaV2.1 (GenBank Accession number M64373), Kir2.1-AAA (15), and GFP-mut3b (16) in pMT2. The CaV2.2-Dom I and YFP-CaV2.2 constructs (12) and the Δ1–55 CaV2.2 truncation (17) have been described previously. Other constructs were made by standard techniques and verified by automated sequencing. They were: CaV2.2 N terminus (residues 1–95), CaV2.1 N terminus (residues 1–100), CaV2.2-Dom I–4TMs (residues 1–225 with a C-terminal myc-His6 tag), CaV2.2-Dom I–4TMs no charges (residues 1–225, with all charged residues in S1–S2, S3–S4 replaced by the noncharged residues: valine, leucine, or isoleucine), CaV2.2-DomI-4TMs no charges-C110S, CaV2.2-Dom I Δ2–91 with an N-terminal SS motif to create a construct starting MstSTEW, CaV2.2-Dom I Δ2–91 and CaV2.2-Dom I Δ1–55. When a CAAX motif was added, this was the C-terminal 10 amino acids of R-Ras (GCMSCKC)VL5. It was added to the C terminus of GFP-mut3b, CaV2.2 N terminus (residues 1–95), CaV2.2 N terminus containing the R52A/R54A mutation, CaV2.2 N terminus (Δ2–42), CaV2.1 N terminus (residues 1–100), and CaV2.1 N terminus R57A/R59A. These were all subcloned into an in-frame Xhol site of the CAAX pmt2 vector, which then creates an additional arginine residue between the construct and CAAX motif.

**Cell Culture and Heterologous Expression**—COS-7 cells were cultured as described previously (18). The TS-A-201 cells were cultured in a medium consisting of Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum, 1% Glutamax, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were transfected using FuGENE 6 (Roche Diagnostics). The cDNAs (all at 1 μg/μl) for CaV α1 subunits, truncated domain constructs, α2δ-1 or α2δ-2, β1b, and when used as a reporter of transfected cells, were mixed in a ratio of 3:1:2.1:0.2, unless stated otherwise. When particular subunits were not used, the volume was made up with water or blank vector, or the volume of transfection reagent was reduced, all with equivalent results.

Dorsal root ganglion (DRG) neurons isolated from Sprague-Dawley rats (175–250 g) in ice-cold Hanks’ balanced salt solution (Invitrogen) were transferred to DMEM nutrient mixture F-12 (DMEM/F12) containing 0.4 mg/ml trypsin, 0.6 mg/ml collagenase type 1 (both from Worthington Biochemical Corp.), and 100 units/ml DNase (Invitrogen), saturated with 95% O2/5% CO2, and incubated for 1 h at 37 °C. The neurons were washed in DMEM/F12 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% Glutamax (10% DMEM). Neurons were dissociated by vigorous shaking, centrifuged twice for 9 min at 800 × g, and the pellet was resuspended in 200 μl of Amaxa rat neuron nucleofector solution (Lonza Cologne AG, Cologne, Germany). Suspended neurons were mixed with cDNA for the truncated domain constructs (40 ng/μl) and YFP (20 ng/μl) DNA and transfected with nucleofector program O-003 following the manufacturer’s instructions. The effect of the constructs was compared with DRG neurons expressing only YFP cDNA. The transfection reagent was neutralized with 500 μl of 10% DMEM supplemented with 50 ng/ml nerve growth factor. Neurons from each group were plated on poly-L-lysine (0.5 mg/ml)-coated 22-mm coverslips (BDH), placed in 35-mm polystyrene tissue culture dishes for 2 h to settle, flooded with 10% DMEM supplemented with 50 ng/ml nerve growth factor, and cultured for 3–4 days at 37 °C. Prior to experiments, the numerous neurite processes were eliminated by replating to improve voltage-clamp recording. Culture medium was removed, and cells were incubated for 5 min at 37 °C in 1 ml of 10% DMEM containing 0.2% type 1 collagenase. The enzymatic reaction was stopped with 1 ml of 10% DMEM, and the neurons were triturated and spun for 9 min at 800 × g. The pellet was resuspended in 300 μl of 10% DMEM, and the cells from each group were plated on poly-L-lysine-coated coverslips and left to recover for at least 2 h at 37 °C before recording.

**Xenopus** oocytes were prepared, injected, and utilized for electrophysiology as described previously (17), with the following exceptions. Plasmid cDNAs for the different calcium channel subunits α1, α2δ, β1b, and truncated or mutated domains and other constructs were mixed in 2:1:2:2 ratios at 1 μg/μl, unless stated otherwise, and 9 nl was injected intranuclearily, after 2-fold dilution of the cDNA mixes. When the truncated domain was not included it was replaced by an equivalent volume of empty vector, water, or a cDNA for a nonfunctional transmembrane protein, Kir-AAA (15) with equivalent results.

**Electrophysiology**—For TS-A-201 cells, the patch pipette solution contained 140 mM cesium aspartate, 5 mM EGTA, 2 mM MgCl2, 0.1 mM CaCl2, 2 mM K2ATP, 10 mM Hepes, pH 7.2, 310 mOsM with sucrose. The external solution contained 150 mM tetraethylammonium bromide, 3 mM KCl, 1.0 mM NaHCO3, 1.0 mM MgCl2, 10 mM Hepes, 4 mM glucose, 1 mM BaCl2, pH 7.4, 320 mOsM with sucrose. For DRGs, the patch pipette solution contained 140 mM cesium aspartate, 10 mM EGTA, 2 mM MgCl2, 5 mM K2ATP, 10 mM Hepes, pH 7.2, 310 mOsM with sucrose. The external solution was identical to that described above, except 10 mM BaCl2 was used, and 1 μM tetrodotoxin was included in the medium to suppress voltage-gated Na+ currents. I0 s was recorded using an Axopatch 1D amplifier (Axon Instruments, Molecular Devices, Sunnyvale CA), and data were filtered at 2 kHz and digitized at 10 kHz. Analysis was performed using pClamp9 (Axon) and Origin 7 (Microcal Origin, Northampton, MA). Current records are shown following leak and residual capacitance current subtraction (P/4 protocol). Incompletely subtracted capacitative transients have been truncated in traces shown. Recordings in Xenopus oocytes were performed as described (19), and all recordings were performed 48–60 h after injection for CaV2.2 and 72–80 h after injection for CaV2.1. The Ba2+ concentration was 10 mM, unless stated otherwise. When stated, current-voltage (I–V) plots were fit with a modified Boltzmann equation as described, for determination of the voltage for 50% activation (19).

**Western Blotting and Calcium Channel Subunit Quantification**—COS-7 cells were processed for SDS-PAGE as described (12). Samples (50 μg of cell lysate protein/lane) were separated using Novex 4–12% Tris-glycine or 4–12% BisTris
NuPAGE gels (Invitrogen) and transferred electrophoretically to polyvinylidene fluoride membranes. The membranes were blocked with 3% bovine serum albumin and 0.02% Tween 20 and then incubated overnight at room temperature with the relevant primary antibody: 1:1000 dilution of anti-CaV2.2 (12). Detection was performed either with a 1:1000 dilution of goat anti-rabbit (or anti-mouse) IgG-horseradish peroxidase conjugate (Bio-Rad) and ECL Plus (Amersham Biosciences), or with a 1:1000 dilution of goat anti-rabbit IgG-Cy5 conjugate (Amersham Pharmacia Biotech), all in conjunction with a Typhoon 9410 Variable Mode Imager (Amersham Pharmacia Biotech), set in chemiluminescence or fluorescence mode, respectively. Protein bands were quantified using ImageQuant 5.2. The same amount of total protein was loaded for all samples on a gel for accurate comparison between lanes.

RESULTS

To extend our studies on the suppression of CaV2.x channels by truncated domains containing Domain I (Fig. 1A) (9, 12), in terms of the specific truncated domain involved, we first mutated various structural motifs in Domain I and expressed the resultant constructs to narrow down the element(s) responsible for the suppression. We have used a number of different expression systems and methods to ensure that our results are able to generalize beyond a single system. Key experiments have been reproduced by more than one method.

Which Structural Elements within Domain I of CaV2 Channels Are Required for Suppression?—CaV2.2-Dom I alone produced ~90% suppression of CaV2.2 currents in Xenopus oocytes (Fig. 1, B and C). CaV2.2-Dom I was previously found to be more effective than CaV2.2-Dom I-II to inhibit CaV2.2 currents (12). We then found that a construct consisting of the NT, the first four TM segments (S1–S4) and the C terminus (12). We then found that a construct consisting of the N terminus and the first four TM segments (S1–S4) of CaV2.2 (CaV2.2-Dom I-4TMs) was as effective as CaV2.2-Dom I, CaV2.2-H11011, Ba was again undiminished (Fig. 1, B and C). A conserved set of structural motifs in this region of CaV2.2-Dom I is the charged amino acids in TM segments S1–S4, which might mediate inappropriate interaction between the full-length and truncated channel. However, mutation of all charged amino acids in the TM segments S1, S2, S3, and S4 to hydrophobic residues, within the truncated CaV2.2-Dom I-4TMs construct, did not significantly affect the ability of this construct to suppress CaV2.2 currents (Fig. 1, B and C). A second potential source of interaction is the conserved cysteines (in S1 and S2) which might form disulfide bonds with the full-length WT channel. However, when the cysteine in S1 (Cys110) was mutated to serine to form CaV2.2-Dom I-4TMs (no charges, C110S), suppression of Iba was again undiminished (Fig. 1, B and C).

Role of the N Terminus of CaV2.2 in Dominant Negative Suppression—We then surmised that the cytoplasmic N terminus might contain structural elements involved in suppression. To examine the role of the N terminus, we utilized truncated and full-length constructs of CaV2.2, in which either or both were engineered to contain N-terminal deletions. We have previously shown that Δ1–55 CaV2.2 produced functional channels (17).

We first utilized expression in tsA-201 cells and compared the ability of two N-terminally deleted, truncated constructs of CaV2.2-Dom I (Δ1–55 CaV2.2-Dom I and Δ2–91 CaV2.2-Dom...
Suppression of Ca\textsubscript{v}2.x Channels by N Terminus

A

![Graph showing suppression of Ca\textsubscript{v}2.x channels by N terminus](image)

B

![Image showing peak Ca\textsubscript{v}2.2 currents](image)

FIGURE 2. Effect of N-terminally truncated Ca\textsubscript{v}2.2 domains on Ca\textsubscript{v}2.2 \(I_{\text{Ba}}\) when expressed in tsA-201 cells. A, peak \(I_{\text{Ba}}\) was determined from \(I-V\) relationships in 1 mM Ba\textsuperscript{2+} following expression in tsA-201 cells. The currents in the presence of the stated truncated domain are expressed as a percentage of control currents in its absence for Ca\textsubscript{v}2.2-\(\Delta\text{I}1-55\) (filled bars) or \(\Delta\text{I}1-55\) Ca\textsubscript{v}2.2-\(\Delta\text{I}1-55\) (open bars). Data were pooled from several experiments, each examining the effect of one truncated construct, and normalized to the respective control in each experiment. The statistical significances of the differences compared with control were determined by Student’s t test; *, \(p<0.05\). The numbers of determinations are given above each bar. Error bars indicate S.E. B, representative current traces (from −30 to +15 mV at 15 mV, from a holding potential of −90 mV), for Ca\textsubscript{v}2.2-\(\Delta\text{I}1-95\) Ca\textsubscript{v}2.2 (upper panel) and \(\Delta\text{I}1-55\) Ca\textsubscript{v}2.2-\(\Delta\text{I}1-55\) (lower panel) in the absence or presence of Ca\textsubscript{v}2.2-Dom I, \(\Delta\text{I}1-55\) Ca\textsubscript{v}2.2-Dom I, or \(\Delta\text{I}2-91\) Ca\textsubscript{v}2.2-Dom I. The scale bars refer to all traces.

I) for their ability to suppress expression of Ca\textsubscript{v}2.2 and \(\Delta\text{I}1-55\) Ca\textsubscript{v}2.2 \(I_{\text{Ba}}\) (Fig. 2A). We found that the two N-terminally truncated Domain I constructs showed consistently less suppression of \(\Delta\text{I}1-55\) Ca\textsubscript{v}2.2 than of Ca\textsubscript{v}2.2 itself (Fig. 2), and there was no suppression of \(\Delta\text{I}1-55\) Ca\textsubscript{v}2.2 by the construct with the longer N-terminal deletion, \(\Delta\text{I}2-91\) Ca\textsubscript{v}2.2-Dom I (Fig. 2).

We then examined whether \(\Delta\text{I}2-91\) Ca\textsubscript{v}2.2 was functional and found that, unlike truncations up to residue 55, its expression did not result in any discernible calcium channel currents (Fig. 3A). We next examined whether this N-terminally truncated channel would suppress expression of the full-length WT Ca\textsubscript{v}2.2 \(I_{\text{Ba}}\) and found substantial inhibition of Ca\textsubscript{v}2.2 currents in Xenopus oocytes (Fig. 3A). The peak Ca\textsubscript{v}2.2 \(I_{\text{Ba}}\) at +5 mV was reduced to 41.4 ± 10.1% of control (\(p=0.016\)) in the additional presence of \(\Delta\text{I}2-91\) Ca\textsubscript{v}2.2. However, there was no effect on any other properties of the currents, including steady-state inactivation (Fig. 3B). A similar result was observed when the same constructs were expressed in tsA-201 cells (Fig. 3C). Taken together, these data suggest that at least one intact N terminus, on either the full-length or the truncated channel construct, is required for suppression; and in agreement with this hypothesis, we found no suppression of \(\Delta\text{I}1-55\) Ca\textsubscript{v}2.2 currents by the \(\Delta\text{I}2-91\) Ca\textsubscript{v}2.2 construct in the same experiment (Fig. 3C).

Dominant Negative Suppression Requires a Free N Terminus—To examine further the role of the N terminus, we then investigated whether its effect could be prevented by addition of a bulky tag attached to the extreme N terminus of Ca\textsubscript{v}2.2. We found that there was no significant inhibition of YFP-Ca\textsubscript{v}2.2 currents by YFP-Ca\textsubscript{v}2.2-Dom I (14.8 ± 11.4% inhibition, Fig. 3D). This result indicates that the presence of an intact free N terminus, unencumbered by the bulky YFP tag, is required for dominant negative suppression.

Construct Consisting of the N Terminus of Ca\textsubscript{v}2.2 Suppresses Ca\textsubscript{v}2.2 Channel Expression—We had previously shown that an N-terminal construct of Ca\textsubscript{v}2.2 did not inhibit GFP-Ca\textsubscript{v}2.2 currents (12). This result was confirmed in the present study, the peak \(I_{\text{Ba}}\) resulting from expression of GFP-Ca\textsubscript{v}2.2 was non-significantly reduced in the presence of the N terminus of Ca\textsubscript{v}2.2 (residues 1–95), by 8.9 ± 19.5% (\(n=13\); Fig. 4A).

In the light of the results described above, we then examined whether a construct consisting of the Ca\textsubscript{v}2.2 cytoplasmic N terminus alone would be capable of inhibiting WT Ca\textsubscript{v}2.2 \(I_{\text{Ba}}\). We found a 40% reduction in WT Ca\textsubscript{v}2.2 currents when the Ca\textsubscript{v}2.2 N terminus was co-expressed (Fig. 4, B and C).

To examine how the Ca\textsubscript{v}2.2 N terminus was inhibiting Ca\textsubscript{v}2.2 currents, we attached an extended CAAX motif to its C terminus, consisting of the last 10 amino acids of H-Ras. This would promote both prenylation and palmitoylation of the polypeptide and thus enhance the concentration associated with both plasma and internal membranes (20). To confirm the localization of constructs to which a CAAX motif was attached, we examined the distribution of GFP-CAAX, which was found to be associated both at the plasma membrane and also with cytoplasmic organelles (Fig. 4D), in contrast to free GFP, which was observed uniformly throughout the cytoplasm and also in the nucleus (Fig. 4D). The membrane-tethered Ca\textsubscript{v}2.2 N terminus-CAAX produced a very strong inhibition of Ca\textsubscript{v}2.2 currents, by 70% at 0 mV, whereas a control prenylated protein (GFP-CAAX) produced no significant inhibition (Fig. 4, B, C, and E).
We then examined whether truncation of the Ca\textsubscript{v}2.2 N terminus would prevent this inhibition and found that no significant inhibition was produced by Ca\textsubscript{v}2.2 N terminus (Δ2–42)-CAA\textsubscript{X} (Fig. 4B), indicating that the extreme N terminus is involved in the process of suppression of Ca\textsubscript{v}2.2 currents. We previously identified an 11-amino acid motif (residues 45–55) in the N terminus of Ca\textsubscript{v}2.x channels, YKQS\textsubscript{X}AQRART, which was essential for G protein-mediated inhibition of these channels (17, 21). Two key amino acids involved in this process were the two arginine residues in this motif. We therefore examined whether the same motif was involved in suppression of Ca\textsubscript{v}2.2 current expression by mutating these two amino acids to alanine in the Ca\textsubscript{v}2.2 N terminus-CAA\textsubscript{X} construct. We found that these mutations prevented the effect of the N terminus on Ca\textsubscript{v}2.2 currents, even producing a small increase compared with Ca\textsubscript{v}2.2 alone (Fig. 4, B and E). Together with the
Suppression of CaV2.2 Channels by N Terminus

A. GFP-CaV2.2

B. % of control I_{Ca}

C. CaV2.2

D. GFP-CAAX

E. GFP-CAAX

F. % of control I_{Ca}

G. 

JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 285 • NUMBER 2 • JANUARY 8, 2010

840
previous result, this suggests that docking of the free N terminus via a motif including Arg^52 and Arg^44 is involved in its ability to suppress CaV_{2.2} currents but that the extreme N terminus of CaV_{2.2} must also play a role.

Further studies showed that co-expression of the CaV_{2.2} N-terminal construct also produced a significant inhibition (~50%) of Δ1–55 CaV_{2.2} I_{\text{N}} (Fig. 4F), and co-expression of CaV_{2.2} N terminus-CAAX strongly inhibited (by 75%) Δ1–55 CaV_{2.2} I_{\text{N}} (Fig. 4F), indicating that the suppressive effect of the CaV_{2.2} N terminus does not require the presence of the same motif on the full-length channel. This provides evidence that the suppressive effect is probably not via dimerization of the N termini.

One potential explanation for the reduction of CaV_{2.2} I_{\text{N}} by the CaV_{2.2} N-terminal construct, given the involvement of the amino acids 45–55 (17, 21), was that there could be tonic modulation of the calcium channel currents (22). However, co-expression of the CaV_{2.2} N-terminal constructs was not associated with slowed activation of the CaV_{2.2} currents (Fig. 4G). This was therefore discarded as a possibility.

Inhibition of CaV_{2.1} by the N Terminus of CaV_{2.1}—We next examined the effect of the N terminus of CaV_{2.1} on the expression of the P/Q-type channel CaV_{2.1}. Co-expression of the CaV_{2.1} N terminus with CaV_{2.1}/\alpha_{\delta}/\beta_{2}/\beta_{4} produced 37.9% inhibition of the peak I_{\text{N}} currents (Fig. 5, A and B). The relevance of this combination of channel subunits is that it is likely to be one of the main channel complexes in cerebellar Purkinje neurons (13), which mediates many of the effects of the mutations in episodic ataxia type 2. The effect of co-expression of the N terminus of CaV_{2.1} attached to a CAAX motif was a 43.8% reduction in peak I_{\text{N}} and this was also reversed by the corresponding mutations in the key arginine residues, Arg^57 and Arg^59 (Fig. 5B).

Furthermore, the CAAX motif-linked N terminus of CaV_{2.2}, which has a strong homology to CaV_{2.1}, also inhibited CaV_{2.1} currents by 63.5%, whereas the mutant R52A/R54A CaV_{2.2} N terminus-CAAX construct produced no inhibition, rather resulting in an increase in the peak I_{\text{N}} compared with CaV_{2.1}/\alpha_{\delta}/\beta_{2}/\beta_{4} alone (Fig. 5C).

Biochemical Basis for Suppression by the CaV_{2.2} N Terminus of CaV_{2.2} Channel Expression—We previously established that dominant negative suppression by truncated constructs involves a reduction of Ca_{\alpha1} subunit protein expression (9, 12). In the present study, we found that co-expression of the CaV_{2.2} N terminus-CAAX with CaV_{2.2}/\beta_{1}\alpha_{\delta}/\beta_{4}/\alpha_{\delta}-1 in tsA-201 cells resulted in a consistent decrease in the level of CaV_{2.2} protein (Fig. 6A). This was normalized to the total amount of protein in each sample and quantified as a 53% reduction, from six separate transfections (Fig. 6B). We also confirmed that expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not altered by these manipulations (supplemental Fig. 1).

In our previous study implicating the unfolded protein response in this process, we also examined the level of co-expressed \alpha_{\delta}-1 protein and found it also to be reduced (9). The level of \alpha_{\delta}-1 was also reduced by co-expression of the CaV_{2.2} N terminus in the present study, by 51% (n = 6; Fig. 6, A and B). To confirm whether the RAR motif in the N terminus was involved in this response, we examined the effect of the R52A/R54A CaV_{2.2} N terminus-CAAX construct. In agreement with our electrophysiological results, the R52A/R54A CaV_{2.2} N terminus-CAAX construct produced no suppression of expression of CaV_{2.2} and \alpha_{\delta}-1 protein (Fig. 6, A and B).

The inclusion of GFP as a marker of transfection and cell survival showed that the effect of CaV_{2.2} N terminus-CAAX was not due to a reduction in transfected cell number at the time of harvesting the cells. The proportion of GFP-positive cells present 48 h after transfection was 4.6 ± 0.5%, 5.8 ± 0.5%, and 4.9 ± 0.5% in tsA-201 cells transfected with CaV_{2.2}/\beta_{1}/\alpha_{\delta}/\beta_{2}/\beta_{4}/\alpha_{\delta}-1/pMT2, CaV_{2.2}/\beta_{1}/\alpha_{\delta}/\beta_{2}/\beta_{4}/\alpha_{\delta}-1/CaV_{2.2} N terminus-CAAX, and CaV_{2.2}/\beta_{1}/\alpha_{\delta}/\beta_{2}/\beta_{4}/R52A/R54A CaV_{2.2} N terminus-CAAX, respectively. These were not significantly different (n = 3, one-way ANOVA and Tukey’s post hoc test).

Is There an Interaction between the N Terminus of CaV_{2.2} and Other Domains of CaV_{2.2}?—We were unable to demonstrate any positive interactions between the N terminus of CaV_{2.2} and the CaV_{2.2} I-II loop, CaV_{2.2} Dom I or a number of other CaV_{2.2} sequences, using the yeast two-hybrid assay (supplemental data), in contrast to a previous study (22). Therefore we cannot identify a high affinity interaction of the CaV_{2.2} N terminus with a particular peptide domain of CaV_{2.2} using this system, indicating that it is perhaps more likely to interact in a complex binding pocket, made up of multiple elements.

Effect of Truncated Calcium Channels on Endogenous Calcium Currents in DRG Neurons—We wished to examine whether the truncated constructs would also affect endogenous calcium channel currents, and we therefore expressed these constructs in DRG neurons using Amaxa transfection.

FIGURE 4. Examination of the effect of the N terminus of CaV_{2.2} on functional expression of CaV_{2.2}. A, example of current traces for voltage steps from −40 mV to +40 mV from a holding potential of −100 mV for GFP-CaV_{2.2}/\alpha_{\delta}/\beta_{2}/\beta_{4}/\alpha_{\delta}-1 alone (left), and together with CaV_{2.2} N terminus (right). Recordings were made with 10 mM Ba^{2+} in Xenopus oocytes. B, peak I_{\text{N}} for CaV_{2.2}/\alpha_{\delta}/\beta_{2}/\beta_{4}/\alpha_{\delta}-1 alone (open bar, n = 36), GFP-CAAX (gray bar, n = 16), CaV_{2.2} N terminus 1–95-CAAX (hashed bar, n = 37), CaV_{2.2} N terminus Δ2–42-CAAX (horizontal striped bar, n = 25), and R52A/R54A CaV_{2.2} N terminus-CAAX (cross-hatched bar, n = 19). The statistical significances of the differences indicated were determined by one-way ANOVA and Bonferroni’s post hoc test. **, p < 0.0016; ***, p < 0.001. Error bars indicate S.E. C, example of current traces for voltage steps from −40 mV to +40 mV for GFP-CaV_{2.2}/\alpha_{\delta}/\beta_{2}/\beta_{4}/\alpha_{\delta}-1/beta_{1}/\beta_{2}/\beta_{4}/\alpha_{\delta}-1/beta_{4} alone (left, and together with CaV_{2.2} N terminus (right). Recordings were made with 10 mM Ba^{2+} in Xenopus oocytes, and co-expressed with GFP-CAAX (■, n = 18), CaV_{2.2} N terminus-CAAX (○, n = 18), or R52A/R54A CaV_{2.2} N terminus-CAAX (△, n = 19). All recordings were performed in parallel using 10 mM Ba^{2+} at 20 mV/s. The I-V relationships for CaV_{2.2}/\alpha_{\delta}/\beta_{2}/\beta_{4}/\alpha_{\delta}-1/beta_{1} expressed in Xenopus oocytes, co-expressed with GFP-CAAX (■, n = 18), CaV_{2.2} N terminus-CAAX (○, n = 18), or R52A/R54A CaV_{2.2} N terminus-CAAX (△, n = 19). All recordings were performed in parallel using 10 mM Ba^{2+}. The I-V curves are fit with a modified Boltzmann relationship, as described under “Experimental Procedures.” The V_{1/2} was −8.6 mV for GFP-CAAX, −7.1 mV for CaV_{2.2} N terminus-CAAX, and −8.4 mV for R52A/R54A CaV_{2.2} N terminus-CAAX. The peak I_{\text{N}} (open bar, n = 36), GFP-CAAX (gray bar, n = 10), and CaV_{2.2} N terminus-CAAX (hashed bar, n = 9). The statistical significances of the differences indicated were determined by Student’s two-tailed t test. ***, p < 0.001; ***, p < 0.001. Recordings were made with 10 mM Ba^{2+}. G, voltage dependence of time constant of activation (τ_{\text{act}}; left) for CaV_{2.2}/\alpha_{\delta}/\beta_{2}/\beta_{4}/\alpha_{\delta}-1/beta_{1} without (●, n = 12) or with (○, n = 7) the free CaV_{2.2} N terminus; and right, for CaV_{2.2}/\alpha_{\delta}/\beta_{2}/\beta_{4}/\alpha_{\delta}-1/beta_{1} with GFP-CAAX (●, n = 10) or with the free CaV_{2.2} N terminus-CAAX (○, n = 13).
We performed experiments 4 days after transfection, to allow synthesis of endogenous channels to occur, and in the presence of 10 μM nifedipine, to isolate native N-type calcium channel currents (Fig. 7, A–C). Expression of CaV2.2 N terminus-CAA produced a statistically significant reduction in DRG \( I_{Ba} \) (Fig. 5, A–C). Expression of CaV2.1 N terminus produced a statistically significant reduction in DRG \( I_{Ba} \) (Fig. 5, A–C). The statistical significances of the differences indicated were determined by Student’s two-tailed \( t \)-test. **, \( p < 0.0046 \); ***, \( p < 0.001 \). Error bars indicate S.E.

**FIGURE 5.** Examination of the role of the N terminus of CaV2.1 on functional expression of CaV2.1. A, mean \( I-V \) relationship for CaV2.1/\( \alpha_\delta \beta-2/\beta 4 \) in Xenopus oocytes, either alone (■, \( n = 15 \)) or co-expressed with CaV2.1 N terminus (○, \( n = 15 \)). All recordings were performed in parallel, using 10 mM Ba\(^{2+} \). The \( I-V \) curves were fit with a modified Boltzmann relationship, up to +35 mV. The \( V_{50,act} \) was −11.9 mV for control and −9.3 mV in the presence of the CaV2.1 N terminus. B, left panel, peak \( I_{Ba} \) (at 0 mV) for CaV2.1/\( \alpha_\delta \beta-2/\beta 4 \) alone (black bar, \( n = 28 \)) or together with CaV2.1 N terminus (open bar, \( n = 26 \)), from two independent experiments, including that depicted in A. Right panel, peak \( I_{Ba} \) for CaV2.1/\( \alpha_\delta \beta-2/\beta 4 \) alone (hatched bar, \( n = 9 \)) or together with CaV2.1 N terminus-CAAX (cross-hatched bar, \( n = 20 \)) or CaV2.1 N terminus R57A/R59A-CAAX (gray bar, \( n = 20 \)). The statistical significances of the differences indicated were determined by Student’s two-tailed \( t \)-test. *, \( p < 0.0162 \); **, \( p < 0.0041 \). Error bars indicate S.E.

**FIGURE 6.** Examination of the effect of the N terminus of CaV2.2 on CaV2.2 protein expression. A, expression of CaV2.2 (upper panel) and \( \alpha_\delta \beta-1 \) (lower panel) protein in untransfected tsA-201 cells (first lane), when CaV2.2/\( \alpha_\delta \beta-1/\beta 1b \) were expressed, alone (second lane) and together with CaV2.2 N terminus-CAAX (third lane), or R52A/R54A CaV2.2 N terminus-CAAX (fourth lane). The same amount of total protein was loaded for all samples on a gel, for accurate comparison among lanes. B, bar chart from quantification of results, including those in A, showing the effect of CaV2.2 N terminus-CAAX (open bars, \( n = 6 \)) or R52A/R54A CaV2.2 N terminus-CAAX (gray bars, \( n = 4 \)) relative to control levels (black bars), for CaV2.2 (left) and \( \alpha_\delta \beta-1 \) (right) protein levels. The statistical significances of the differences indicated were determined by Student’s \( t \)-test. *, \( p < 0.0162 \); **, \( p < 0.0041 \). Error bars indicate S.E.

We performed experiments 4 days after transfection, to allow synthesis of endogenous channels to occur, and in the presence of 10 μM nifedipine, to isolate native N-type calcium channel currents (Fig. 7, A–C). Expression of CaV2.2 N terminus-CAAX produced a statistically significant reduction in DRG \( I_{Ba} \) (Fig. 7, A–C).
Suppression of Ca\textsubscript{v}\textsubscript{2.2} Channels by N Terminus

Here, we have examined the process of dominant negative suppression of Ca\textsubscript{v}\textsubscript{2.1} and Ca\textsubscript{v}\textsubscript{2.2} currents by truncated Ca\textsubscript{v}\textsubscript{2.2} constructs, which was identified by Raghib et al. (12). Our previous conclusion was that if Ca\textsubscript{v}\textsubscript{2.2} is co-expressed with truncated constructs of Ca\textsubscript{v}\textsubscript{2.2} containing Domain I, expression of the full-length Ca\textsubscript{v}\textsubscript{2.2} channel protein is almost completely prevented. We subsequently identified that there was a requirement for interaction between the full-length and truncated constructs (9), which has been confirmed and extended by others (10).

We previously observed cross-suppression between the different subclasses of Ca\textsubscript{v}\textsubscript{2} channels (Ca\textsubscript{v}\textsubscript{2.1}, 2.2, 2.3), where conservation both within the TM segments and in the cytoplasmic N and C termini and loops is fairly high. However, there was no significant cross-suppression between full-length Ca\textsubscript{v}\textsubscript{3.1} and truncated constructs of Ca\textsubscript{v}\textsubscript{2.2}, and vice versa (9). This suggests that the response is induced by the association of part of the truncated domain with segments of a cognate full-length channel with which it shows an affinity. Our present results identify specific motifs involved in the interaction.

We have found that one of the main regions involved in interaction is the N terminus of these channels. We observed that the optimum requirements for this to occur are that the N terminus should be both full-length and have a free N terminus (i.e. not tagged with XFP). When these conditions are met, in either or both of the truncated and the full-length Ca\textsubscript{v}\textsubscript{2.2} channels, suppression occurs, but when the extreme N terminus is missing from both constructs, or both are tagged with XFP, suppression is markedly reduced or absent.

Our finding that an N-terminal XFP tag hinders the dominant negative suppression process may also explain some anomalies in the literature, where such tagged constructs have been used in the study of this phenomenon (7, 23).

Amino acids 1–55 of Ca\textsubscript{v}\textsubscript{2.2} contain the N-terminal motif MVRFGDEL attached to a highly flexible region GGRYGGTG-GGERARGGAGGAGGPGQGGLPPG, representing amino acids 9–40 and identified as being glycine-rich (56%) and hence of low complexity. This region is followed by YKQSIAQRART, which is the 11-amino acid motif that we have previously identified to be essential for G protein modulation in Ca\textsubscript{v}\textsubscript{x} channels. This motif is highly conserved in the Ca\textsubscript{v}\textsubscript{2} family (17, 21) and predicted to form an \(\alpha\)-helix (PSIPRED 2.6). An additional
motif residing within amino acids 56–95 (MALYNPIPVKQN-CFTVNRSLFVFSEDNVVRKYAKRITEWPPFE) was also identified as being involved in the role of the N terminus of CaV2.2 to mediate G protein modulation by Gβγ (22). We have now found that this region is essential for functional expression of the channel.

A possible scenario is that the high mobility of the low complexity region in the N terminus will allow the N terminus to interact with a distant binding pocket on the channel and that the motif containing the key amino acids Arg⁵² and Arg⁵⁴ is involved in this process. This intramolecular interaction might be required as a quality check point for correct folding during channel synthesis. RXR motifs have previously been identified to regulate endoplasmic reticulum retention and retrieval in other channels (24) and may be acting by a similar mechanism here.

In agreement with this hypothesis, our results show that the CaV2.x N terminus, particularly when attached C-terminally to a CAAX motif, results in strong suppression of CaV2.2 expression, both in terms of functional currents and at the level of CaV2.2 protein. This suppression by the N terminus is prevented when the N terminus is truncated and when the amino acids Arg⁵² and Arg⁵⁴ are mutated to Ala. We hypothesize that once the N terminus, either as a free domain or attached to a truncated channel, has interacted intermoleurally with the full-length channel, the misfolded aggregate may both be directed to the proteasomal pathway as suggested by others (10, 25) and may also trigger the unfolded protein response to suppress further translation (9, 26).

The relevance to episodic ataxia type 2 is that many of the mutations found in this dominant disease result in premature protein truncation, but in all the mutations to date, the N terminus is intact, the first known mutation being in Domain I (6). A number of studies have shown previously that the truncated channels predicted by episodic ataxia type 2 mutations interact with the full-length channel (9–11, 23). Here, we have identified that an intact N terminus is essential for interaction between the truncated domain and the full-length channel. Future work to discover the site of interaction may now allow the development of therapeutic agents that hinder this process.

REFERENCES

1. Catterall, W. A. (2000) Annu. Rev. Cell Dev. Biol. 16, 521–555
2. Ertel, E. A., Campbell, K. F., Harpold, M. M., Hofmann, F., Mori, Y., Perez-Reyes, E., Schwartz, A., Snutch, T. P., Tanabe, T., Birnbaumer, L., Tsien, R. W., and Catterall, W. A. (2000) Neuron 25, 533–535
3. Pietrobon, D. (2002) Mol. Neurobiol. 25, 31–50
4. Ophoff, R. A., Terwindt, G. M., Vergouwe, M. N., van Eijk, R., Oeffner, P. J., Hoffman, S. M., Lamerdin, J. E., Mohrenweiser, H. W., Bulman, D. E., Ferrari, M., Haan, J., Lindhout, D., van Ommen, G. J., Hofker, M. H., Ferrari, M. D., and Frants, R. R. (1996) Cell 87, 543–552
5. Denier, C., Ducros, A., Vahedi, K., Joutel, A., Thierry, P., Ritz, A., Castellano, G., Deonna, T., Gérard, P., Devoize, J. L., Gayou, A., Perreouty, B., Soisson, T., Autret, A., Warter, J. M., Vighetto, A., Van Bogaert, P., Alamowitch, S., Rouliet, E., and Tournier-Lasserve, E. (1999) Neurology 52, 1816–1821
6. Jen, J. C., Graves, T. D., Hess, E. J., Hanna, M. G., Griggs, R. C., and Baloh, R. W. (2007) Brain 130, 2484–2493
7. Wappl, E., Koschak, A., Poteser, M., Sinnegger, M. J., Walter, D., Eberhart, A., Groschner, K., Glossmann, H., Kraus, R. L., Grabner, M., and Striessnig, J. (2002) J. Biol. Chem. 277, 6960–6966
8. Maquat, L. E. (2002) Curr. Biol. 12, R196–R197
9. Page, K. M., Heblich, F., Davies, A., Butcher, A. J., Leroy, J., Bertaso, F., Pratt, W. S., and Dolphin, A. C. (2004) J. Neurosci. 24, 5400–5409
10. Mezghrani, A., Monteil, A., Watschinger, K., Sinnegger-Brauns, M. J., Barrière, C., Bourinet, E., Nargeot, J., Striessnig, J., and Lory, P. (2008) J. Neurosci. 28, 4501–4511
11. Jeng, C. J., Chen, Y. T., Chen, Y. W., and Tang, C. Y. (2006) Am. J. Physiol. Cell Physiol. 290, C1209–C1220
12. Raghib, A., Bertaso, F., Davies, A., Page, K. M., Meir, A., Bogdanov, Y., and Dolphin, A. C. (2001) J. Neurosci. 21, 8495–8504
13. Barclay, J., Balaguero, N., Mione, M., Ackerman, S. L., Letts, V. A., Brodbeck, J., Canti, C., Meir, A., Page, K. M., Kusumi, K., Perez-Lander, E., Frankel, W. N., Gardiner, R. M., Dolphin, A. C., and Rees, M. (2001) J. Neurosci. 21, 6095–6104
14. Tomlinson, W. J., Stea, A., Bourinet, E., Charnet, P., Nargeot, J., and Snutch, T. P. (1993) Neuropharmacology 32, 1117–1126
15. Tinker, A., Jan, Y. N., and Jan, L. Y. (1996) Cell 87, 857–868
16. Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) Gene 173, 33–38
17. Canti, C., Page, K. M., Stephens, G. J., and Dolphin, A. C. (1999) J. Neurosci. 19, 6855–6864
18. Campbell, V., Berrow, N., Brickley, K., Page, K., Wade, R., and Dolphin, A. C. (1995) FEBS Lett. 370, 135–140
19. Canti, C., Davies, A., Berrow, N. S., Butcher, A. J., Page, K. M., and Dolphin, A. C. (2001) Biophys. J. 81, 1439–1451
20. Choy, E., Chiu, V. K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I. E., and Philips, M. R. (1999) Cell 98, 69–80
21. Page, K. M., Canti, C., Stephens, G. J., Berrow, N. S., and Dolphin, A. C. (1998) J. Neurosci. 18, 4815–4824
22. Agler, H. L., Evans, J., Tay, I. H., Anderson, M. J., Colecraft, H. M., and Yue, D. T. (2005) Neuron 46, 891–904
23. Jeng, C. J., Sun, M. C., Chen, Y. W., and Tang, C. Y. (2008) J. Cell. Physiol. 214, 422–433
24. Schwappach, B., Zerangue, N., Jan, Y. N., and Jan, L. Y. (2000) Neuron 26, 155–167
25. Chevet, E., Cameron, P. H., Pelletier, M. F., Thomas, D. Y., and Bergeron, J. J. M. (2001) Curr. Opin. Struct. Biol. 11, 120–124
26. Ron, D. (2002) J. Clin. Invest. 110, 1383–1388