Dominant-negative effects of human P/Q-type Ca\(^{2+}\) channel mutations associated with episodic ataxia type 2

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Jeng, Chung-Jiuan, Yu-Ting Chen, Yi-Wen Chen, and Chih-Yung Tang. Dominant-negative effects of human P/Q-type Ca\(^{2+}\) channel mutations associated with episodic ataxia type 2. Am J Physiol Cell Physiol 290: C1209–C1220, 2006. First published November 23, 2005; doi:10.1152/ajpcell.00247.2005.—Episodic ataxia type 2 (EA2) is an inherited autosomal dominant disorder related to cerebellar dysfunction and is associated with mutations in the pore-forming \(\alpha_{1A}\)-subunits of human P/Q-type Ca\(^{2+}\) channels (Cav2.1 channels). The majority of EA2 mutations result in significant loss-of-function phenotypes. Whether EA2 mutants may display dominant-negative effects in human, however, remains controversial. To address this issue, five EA2 mutants in the long isoform of human \(\alpha_{1A}\)-subunits were expressed in Xenopus oocytes to explore their potential dominant-negative effects. Upon coexpressing the cRNA of \(\alpha_{1A}\)-WT with each \(\alpha_{1A}\)-mutant in molar ratios ranging from 1:1 to 1:10, the amplitude of Ba\(^{2+}\) currents through wild-type (WT)-Cav2.1 channels decreased significantly as the relative molar ratio of \(\alpha_{1A}\)-mutants increased, suggesting the presence of an \(\alpha_{1A}\)-mutant-specific suppression effect. When we coexpressed \(\alpha_{1A}\)-WT with proteins not known to interact with Cav2.1 channels, we observed no significant suppression effects. Furthermore, increasing the amount of auxiliary subunits resulted in partial reversal of the suppression effects in nonsense but not missense EA2 mutants. On the other hand, when we repeated the same coinjection experiments of \(\alpha_{1A}\)-WT and mutant using a splice variant of \(\alpha_{1A}\)-subunit that contained a considerably shorter COOH terminus (i.e., the short isoform), no significant suppression effects were noted until we enhanced the relative molar ratio to 1:10. Altogether, these results indicate that for human WT-Cav2.1 channels comprising the long-\(\alpha_{1A}\)-subunit isoform, both missense and nonsense EA2 mutants indeed display prominent dominant-negative effects.

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The purpose of this study was to test the hypothesis that EA2 mutants might exert dominant-negative effects on human Cav2.1 channels. We systematically assessed the functional interaction between five EA2 mutants and the human WT α1A-subunit. Our results strongly suggest that for both missense and nonsense mutations, EA2 phenotypes may occur as a result of dominant-negative effects. The potency of the EA2 suppression effect varies significantly between two splice variants of human α1A-subunits, however.

MATERIALS AND METHODS

Our animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC approval no. 20030120) of the National Taiwan University College of Medicine and College of Public Health.

Molecular biology. Full-length cDNA for all constructs of the long isoform of human α1A-subunits (GenBank accession no. AF004884), as well as those for α2δ- and β1-subunits, were kindly prepared and generously provided by Dr. Joerg Striessnig (Dept. of Pharmacology and Toxicology, University of California, Los Angeles, Los Angeles, CA) (22). In human cerebellum, approximately two-thirds of the α1A-subunit splice variant is the long isoform (44). As indicated in RESULTS, in one line of experimentation, we also used the short isoform of the human α1A-subunit (equivalent to GenBank accession no. AF004883, but with one splice deletion of tripeptide VEA) (56), which was kindly provided by Dr. Jörg Striessnig (Dept. of Pharmacology and Toxicology, Institute of Pharmacology, University of Innsbruck, Innsbruck, Austria).

To avoid confusion, we adopted recently published nomenclature for the location of different mutations in α1A-subunits (21). To increase the level of protein expression in Xenopus oocytes, cDNA for the α1A-subunit (long isoform) and the α2δ-subunit were subcloned using BamH1 and XbaI sites into the pGEMHE vector kindly provided by Dr. Emily Liman (University of Southern California, Los Angeles, Los Angeles, CA) (29).

For in vitro transcription, cDNA was linearized with XbaI (for α1A- and α2δ-subunits) or with HindIII (for β1-subunits). Capped cRNA was transcribed in vitro from the linearized cDNA template using the mMessage mMachine T7 kit (Ambion, Austin, TX). Concentration of cRNA was determined using gel electrophoresis and verified using spectrophotometry (GeneQuant Pro RNA/DNA calculator; Amersham Biosciences, Piscataway, NJ).

cRNA injection in Xenopus oocytes. Adult female Xenopus laevis (African Xenopus Facility, Knysna, South Africa) were anesthetized by immersion in Tricaine (1.5 g/l). Ovarian follicles were removed from Xenopus frogs, cut into small pieces, and incubated in ND96 solution (in mM: 96 NaCl, 2 KCl, 1.8 MgCl2, 1.8 CaCl2, and 5 HEPES, pH 7.2). To remove the follicular membrane, Xenopus oocytes were incubated in Ca2+-free ND96 containing collagenase (2 mg/ml) on an orbital shaker (~200 rpm) for ~60~90 min at room temperature. After being washed several times with collagenase-free, Ca2+-free ND96, Xenopus oocytes were transferred into ND96 solution. Stage V-VI Xenopus oocytes were then selected for cRNA injection.

For all cRNA injection paradigms, the total volume of injection was always 41.4 nl/oocyte. To examine the phenotype of WT-Cav2.1 or mutant human Cav2.1 channels, Xenopus oocytes were injected with a 1:1:1 molar ratio combination of α1A, α2δ, and β1 cRNA up to a maximal cRNA amount of 81 ng/oocyte. For coexpression experiments, it was imperative to find a submaximal cRNA concentration for WT-Cav2.1 channels that allowed us to add an extra amount of cRNA for mutant α1A-subunits. Therefore, we empirically set up a fixed concentration of cRNA for injection, known as the standard cRNA cocktail, in which α1A-, α2δ-, and β1-cRNA were mixed in a molar ratio of 1:2:2, and the final injection amounts for α1A-, α2δ-, and β1-cRNA were ~3.8 ng, ~3.8 ng, and ~1.9 ng/Xenopus oocyte, respectively. When necessary, a sixfold dilution of the standard cRNA cocktail, which we refer to as the low-cRNA cocktail, was instead used for Xenopus oocyte injection. Injected Xenopus oocytes were stored at 16°C in ND96 and functionally assayed 2–5 days after cRNA injection.

Electrophysiology and data analysis. For functional studies, Xenopus oocytes were transferred into a recording bath containing Cl−-free Ba2+-solution of the following composition (in mM): 40 Ba(OH)2, 50 NaOH, 2 CsOH, and 5 HEPES (pH 7.4 with methanesulfonic acid). To minimize the contribution of endogenous Ca2+-activated Cl− currents, niflumic acid (0.4 mM), which has been shown to display potent blocking effects on endogenous Ca2+-activated Cl− currents in Xenopus oocytes (58), was added to the bath solution. The removal of contaminating Cl− currents by niflumic acid was verified by the suppression of slow inward tail currents during repolarization after a depolarizing test pulse (6). The bath volume was ~200 μl. An agarose bridge was used to connect the bath solution to a ground chamber (containing 3 M KCl), into which two ground electrodes were inserted. Both capillary electrodes (0.1–1 MΩ) used in voltage recording and current injection were filled with 3 M KCl. Ba2+-currents through Cav2.1 channels were acquired using the conventional two-electrode voltage-clamp technique with an OC-725C oocyte clamp (Warner Instrument, Hamden, CT). Data were filtered at 1 kHz (OC-725C oocyte clamp) and digitized at 100 μs per point using the Digidata 1332A/pCLAMP 8.0 data acquisition system (Axon Instruments, Foster City, CA). The holding potential was set at ~90 mV, and 70-mV test pulses were typically applied in 10-mV increments from ~80 to ~70 mV. Passive membrane properties were compensated using the P/4 leak subtraction method provided with pCLAMP 8.0 software. All recordings were performed at room temperature (20–22°C).

Data analyses were performed using built-in analytical functions of pCLAMP 8.0 software. Peak Ba2+-current amplitudes measured at each test voltage level were plotted against voltage (current-voltage, or I-V, curve). Apparent reversal potentials (Vrev) were then determined by extrapolating the I-V curve to the voltage axis (typically ~65 mV), and macroscopic channel conductance (G) at each test potential was calculated using the equation: $G = I/(V - Vrev)$, where $I$ is the peak current amplitude and $V$ is the test potential. The voltage dependence of activation was determined by fitting conductance-voltage (G-V) curves using a simple Boltzmann equation: $G/G_{max} = 1/[1 + exp((V_{max} - V)/k)]$, where $G_{max}$ is the maximum conductance, $V_{max}$ is the half-maximal voltage for activation, and $k$ is the slope factor of the G-V curve.

All data are means ± SE. The significance of differences between two means was analyzed using Student’s t-test, whereas means from multiple groups were compared using one-way ANOVA. All statistical analyses were performed with Origin 7.0 software (Microcal, Northampton, MA).

RESULTS

Functional expression of wild-type and mutant α1A-subunits in Xenopus oocytes. We focus on five EA2-related mutant α1A-subunits (α1A-mutants) that have been reported previously. Three of them involve nonsense mutations that introduce a premature stop codon at the site of mutation (R1281X, R1549X, or R1669X) (23, 24, 62), whereas the other two are missense mutations (P1406C and E1761K) (8, 22). As shown in Fig. 1A, all five mutations occurred in the homologous domains 3 and 4 region of the α1A-subunit. The functional properties of some of these mutants have been studied before using cell line expression systems such as human embryonic kidney (HEK) cells. However, an EA2 mutant that is nonfunctional when expressed in a HEK-derived cell line was found to exhibit detectable Ba2+-currents upon expression in Xenopus oocytes (56). We therefore reexamined the functional pheno-
the missense mutation F1406C produced dramatically reduced currents in *Xenopus* oocytes. The other missense mutation (E1761K), however, exhibited no significant Ba\(^{2+}\) currents in *Xenopus* oocytes. To the best of our knowledge, the present study is the first functional study to demonstrate that the E1761K mutation renders human Cav2.1 channels non-functional. Figure 1C shows the scatterplot of the amplitudes of Ba\(^{2+}\) currents at +20 mV recorded in oocytes expressing WT-Cav2.1 or mutant Cav2.1 channels. No detectable currents in *Xenopus* oocytes were observed in the three nonsense mutations (Fig. 1C), which is the typical phenotype expected of truncation mutations. Thus our functional expression studies show that all five α1\(A\)-mutants under investigation resulted in a significant loss of channel function in *Xenopus* oocytes.

Because EA2 is an autosomal dominant disease, most patients with EA2 are expected to carry the heterozygous CACNA1A genotype: one copy each of the WT and mutant allele. Regarding the molecular mechanism of EA2, a loss-of-function phenotype of the mutant CACNA1A gene certainly supports haploinsufficiency as a reasonable mechanism causing the neurological disorder. It is likely, however, that α1\(A\)-mutants may influence the proper function of α1\(A\)-WT negatively by, for example, disrupting channel assembly by competing for the binding of auxiliary subunits, which are known to upregulate the functional expression of voltage-gated Ca\(^{2+}\) channels in the heterologous expression system (2, 50). We therefore wanted to explore the possibility that dominant-negative effects may serve as a major molecular mechanism underlying EA2.

Coexpression of equimolar ratios of α1\(A\)-WT and α1\(A\)-mutants in *Xenopus* oocytes. By definition, pure haploinsufficiency effects are expected of nonfunctional α1\(A\)-mutants that display neither functional nor structural interactions with α1\(A\)-WT. Accordingly, upon coexpressing α1\(A\)-WT and α1\(A\)-mutant in a 1:1 molar ratio in *Xenopus* oocytes, a significant reduction of the Ca\(^{2+}\) channel function (e.g., amplitude of Ba\(^{2+}\) currents) relative to that of expressing α1\(A\)-WT alone would support the idea that the α1\(A\)-mutant possesses dominant-negative effects. On the other hand, if the current amplitude of α1\(A\)-WT turned out not to be affected significantly by the presence of the α1\(A\)-mutant, it would be counted as evidence against dominant-negative effects. We therefore wanted to perform experiments to assess the coexpression of α1\(A\)-WT and α1\(A\)-mutant.

We first empirically established a standard cRNA concentration for *Xenopus* oocyte injection, which we called the standard cRNA cocktail (see MATERIALS AND METHODS), to ensure that the amplitude of Ba\(^{2+}\) currents through human WT-Cav2.1 channels would be large enough to conduct detailed analyses. In the standard cRNA cocktail, a fixed concentration of cRNA for α1\(A\)-WT, α2\(D\)-, and β3-subunits was mixed in a 1:2:2 molar ratio before being injected into oocytes. In other words, the standard cRNA cocktail can be regarded as a control for haploinsufficiency effects, because it is equivalent to coexpressing α1\(A\)-WT with a hypothetical nonfunctional α1\(A\)-mutant that does not exert dominant-negative effects. To evaluate the dominant-negative effects of the five EA2 mutants, *Xenopus* oocytes were coexpressed with α1\(A\)-WT and α1\(A\)-mutant cRNA mixed at a molar ratio of 1:1 (i.e., one copy of cRNA for α1\(A\)-mutant was added to standard cRNA cocktail). As stated above, for all cRNA injection paradigms, the total volume of injection per oocyte was identical (41.4 nl).
The representative data shown in Fig. 2A indicate that the amplitude of Ba\(^{2+}\) currents recorded from *Xenopus* oocytes coexpressing \(\alpha_{1A}\)-WT with any of the five EA2 mutants was significantly smaller than that for oocytes expressing \(\alpha_{1A}\)-WT alone. Because it is not uncommon to observe a wide variation in the amplitude of Ba\(^{2+}\) currents through WT-Cav2.1 channels among different oocytes (see Fig. 1C), we adopted a normalization procedure to perform an objective comparison of the expression levels of Cav2.1 channels. With the same batch of oocytes on the same day of experimentation, the average value of the peak Ba\(^{2+}\) current amplitudes at +20 mV was calculated in 10 or more oocytes expressing WT-Cav2.1 channels. The average value was then used to normalize the peak +20-mV current amplitudes measured in *Xenopus* oocytes subjected to different coinjection paradigms. Normalized data from different batches of *Xenopus* oocytes on different dates were later pooled for comprehensive analysis. In addition, to avoid biased data due to unhealthy *Xenopus* oocytes, we analyzed only data collected during experiments in which there was no apparent difference between different injection paradigms regarding the viability of *Xenopus* oocytes. As shown in Fig. 2B, the presence of nonsense and missense EA2 mutants, the current amplitude of WT-Cav2.1 channels decreased by \(\sim60 – 80\%\), suggesting that the functional expression of WT-Cav2.1 channels was significantly lower in the presence of \(\alpha_{1A}\)-mutants.

Because the gating of Cav2.1 channels is determined by the membrane potential \(V_m\) experienced by the voltage sensors located in \(\alpha_{1A}\)-subunits, the reduction in Ba\(^{2+}\) currents might have arisen from the alteration of the voltage dependence of activation of \(\alpha_{1A}\)-WT by the EA2 mutants. To examine this possibility, we compared the steady-state activation property (conductance-voltage curve, or \(G-V\) curve) of Cav2.1 channels. As shown in Fig. 3 and Table 1, no significant shift in the \(G-V\) curves was observed in the absence or presence of \(\alpha_{1A}\)-mutants, consistent with the idea that the EA2 mutants failed to affect the voltage sensitivity of WT-Cav2.1 channels. Likewise, other biophysical properties of human Cav2.1 channels, such as activation and inactivation kinetics, were not significantly affected by the presence of the EA2 mutants (data not shown).

Therefore, coexpression of \(\alpha_{1A}\)-WT with any of the five EA2 mutants under investigation resulted in significant suppression of the current amplitudes of WT-Cav2.1 channels that cannot be explained by a haploinsufficiency effect, which is consistent with the idea that the EA2 mutants may exert dominant-negative effects.

**Increasing the relative molar ratios of \(\alpha_{1A}\)-mutants reveals significant dominant-negative effects of EA2 mutations.** The decreased amplitude of Cav2.1 currents in the presence of
**Table 1. Steady-state voltage-dependence property of wild-type Cav2.1 channels in the absence or presence of equimolar coexpressions of α1A mutants**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>V0.5</th>
<th>k</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>28</td>
<td>3.5±1.9</td>
<td>4.5±0.3</td>
</tr>
<tr>
<td>WT + R1281x</td>
<td>17</td>
<td>5.8±1.7</td>
<td>4.6±0.5</td>
</tr>
<tr>
<td>WT + E1406K</td>
<td>12</td>
<td>4.1±1.1</td>
<td>4.1±0.4</td>
</tr>
<tr>
<td>WT + E1549x</td>
<td>15</td>
<td>3.9±1.6</td>
<td>4.9±0.7</td>
</tr>
<tr>
<td>WT + E1679x</td>
<td>13</td>
<td>2.8±0.9</td>
<td>5.1±0.3</td>
</tr>
<tr>
<td>WT + E1761K</td>
<td>16</td>
<td>3.9±1.3</td>
<td>4.7±0.9</td>
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</table>

Values are means ± SE; n, no. of oocytes analyzed for each coexpression paradigm. WT, wild type. The conductance-voltage (G-V) curve of each oocyte expressing Cav2.1 currents was fitted with a simple Boltzmann equation: 

\[ V = \frac{V_0.5 - V}{\exp[k(V - V_0.5)]} \]

where \( V_0.5 \) is the half-maximal voltage for activation, and \( k \) is the slope factor of the G-V curve.

\( \alpha_{1A} \)-mutants presumably reflect an \( \alpha_{1A} \)-mutant-specific suppression of the functional expression of WT-Cav2.1 channels. If there is indeed an \( \alpha_{1A} \)-mutant-specific suppression effect, an increase in the relative molar ratio \( \alpha_{1A} \)-mutant should enhance this suppression effect. We therefore addressed this issue by testing the effect of coexpressing \( \alpha_{1A} \)-WT and \( \alpha_{1A} \)-mutants in the molar ratios of 1:1, 1:3, 1:5, and 1:10 by increasing the amount of \( \alpha_{1A} \)-mutant cRNA added to the standard cRNA cocktail. For all cRNA injection paradigms, the total volume of injection per oocyte was identical (41.4 nl).

Figure 4A shows the representative Ba\(^{2+} \) currents recorded from *Xenopus* oocytes coinjected with different molar ratios of cRNA for WT and R1281x \( \alpha_{1A} \)-subunits, clearly demonstrating that R1281x mutant decreased the expression of WT-Cav2.1 channels in a dose-dependent manner. Compared with the mean current amplitude at +20 mV for the expression of \( \alpha_{1A} \)-WT alone, coexpression with the \( \alpha_{1A} \)-mutant in the molar ratios of 1:1, 1:3, 1:5, and 1:10 resulted in normalized amplitudes of ~36%, ~21%, ~16%, and ~14%, respectively (Fig. 4B), indicating that the amplitude of Ba\(^{2+} \) currents decreased as the relative molar ratio of R1281x increased. The presence of an increased amount of R1281x did not result in any significant shift in the current-voltage relationship (i.e., \( I-V \) curves) of Ba\(^{2+} \) currents (Fig. 4C), suggesting that the foregoing reduction of Ba\(^{2+} \) current was not due to a change in the voltage dependence of WT-Cav2.1 channels. In addition, there was no apparent difference in the viability of oocytes between different coinjection paradigms (data not shown), suggesting that the observed decline in Cav2.1 channel expression did not arise from a deterioration of the viability.

![Figure 4](http://apjpcell.physiology.org/)

**Fig. 4. Effects of enhanced coexpression of R1281x mutant on current amplitudes of WT-Cav2.1 channels in Xenopus oocytes.**

**A:** representative Ba\(^{2+} \) currents recorded from *Xenopus* oocytes coexpressing different molar ratios of R1281x mutant. To achieve indicated molar ratios of \( \alpha_{1A} \)-WT and \( \alpha_{1A} \)-mutant, an appropriate amount of cRNA for R1281x was added to standard cRNA cocktail for oocyte injections. Holding potential was ~90 mV. Pulse protocol comprised 70-ms depolarizing test pulses ranging from ~60 to +50 mV in 10-mV increments. **B:** normalized mean Ba\(^{2+} \) current amplitudes of WT-Cav2.1 channels in the presence of different molar ratios of R1281x. Data were normalized to mean +20-mV Ba\(^{2+} \) current amplitude of WT-Cav2.1 channels in the absence of R1281x (1:0) using the procedure described in Fig. 2B. Mean current amplitudes of coexpressing \( \alpha_{1A} \)-WT and R1281x in the molar ratios 1:1, 1:3, 1:5, and 1:10 were significantly different, \( P < 0.05; \) 1-way ANOVA. Furthermore, when individually compared with coexpression ratio 1:1, mean current amplitude for molar ratio 1:3, 1:5, or 1:10 was significantly smaller (\( * \ P < 0.05; \) t-test), indicating that the amplitude of Ba\(^{2+} \) currents decreased as relative molar ratio of R1281x increased. Number of oocytes measured for each coexpression paradigm is shown in parentheses. **C:** normalized \( I-V \) curves of WT-Cav2.1 channels in the presence of different molar ratios of mutation R1281x. Peak Ba\(^{2+} \) current amplitude measured at each test voltage was normalized to mean current amplitude at +20 mV of WT-Cav2.1 channels. Increased molar coexpression of R1281x failed to shift position of \( I-V \) curves of Cav2.1 channels significantly along the voltage axis.

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of *Xenopus* oocytes as a result of an increased amount of injected cRNA.

Similar suppression effects on α1A-WT were also observed in the other nonsense as well as missense mutants (Fig. 5). For instance, with the F1406C mutant, the normalized mean current amplitude at +20 mV decreased from ~38% at a 1:1 coexpression ratio to only 3% at a 1:10 coexpression ratio. Likewise, with the R1669x mutant, the mean current amplitude at 1:1 and 1:10 molar ratios was ~22% and ~4%, respectively. Increased molar coexpression of the EA2 mutants did not significantly shift the position of the I-V curves of Cav2.1 channels along the voltage axis (data not shown).

An alternative explanation for the suppression effects of the EA2 mutants discussed above is the exhaustion of the cellular machinery for protein synthesis as a result of excessive amounts of cRNA injected into *Xenopus* oocytes. To address this issue, we repeated the experiments by coexpressing WT-Cav2.1 channels with two different types of proteins: densin-180 and rat olfactory channels (ROLF). Densin-180 is a 180-kDa transmembrane protein that is tightly associated with postsynaptic density in neurons and is postulated to function as a synaptic adhesion molecule (1, 55). As demonstrated in Fig. 6A, the average size of Ba\(^{2+}\) currents through WT-Cav2.1 channels did not decrease significantly in the presence of an excessive amount of densin-180. Because all previously reported heterologous expression of densin-180 involved only mammalian expression systems, one might argue that the lack of suppression effect reported herein could be interpreted as a failure of the functional expression of densin-180 in *Xenopus* oocytes. In light of this possibility, we performed the coexpression experiment using ROLF, which is a cyclic nucleotide-gated ion channel that can be expressed functionally in oocytes (48). As shown in Fig. 6B, the mean current amplitude of WT-Cav2.1 channels did not decrease significantly in the presence of an excessive amount of ROLF. Altogether, these results are consistent with the idea that coexpression of α1A-WT and α1A-mutants fail to deplete the cellular machinery for protein synthesis in *Xenopus* oocytes.

Another possibility to explain the suppression effects of the EA2 mutants is that in *Xenopus* oocytes an α1A-subunit-specific suppression effect exists in either the cellular machinery for protein synthesis or the posttranslational processing of Cav2.1 channels per se; that is, both α1A-WT and α1A-mutant may confer this suppression effect. To test this hypothesis, perhaps we should have evaluated the effect of adding up to a 10-fold extra amount of α1A-WT cRNA for oocyte injection. However, because the amount of α1A-WT cRNA was rather high in the standard cRNA cocktail, the viability of *Xenopus* oocytes significantly deteriorated by merely doubling the amount of α1A-WT cRNA (equivalent to 1:1 coinjection ratio),
presumably as a consequence of Ca\textsuperscript{2+}-mediated cytotoxic effects resulting from overexpression of WT-Cav2.1 channels in *Xenopus* oocytes. We therefore performed this experiment using the low-cRNA cocktail for *Xenopus* oocyte injection, in which the amount of cRNA for α1A-, αδ-, and β\textsubscript{4}--subunits was only one-sixth that used for the standard cRNA cocktail. As shown in Fig. 7A, at low-cRNA concentration, increasing the amount of α1A-WT cRNA injected into *Xenopus* oocytes did not lead to suppression of Cav2.1 currents. Instead, the expression of Cav2.1 channels increased. For example, when we enhanced the amount of cRNA for α1A-WT sixfold (coinjection ratio of 1:5), which is equivalent to the amount of α1A-WT in the standard cRNA cocktail, the mean amplitude of Ba\textsuperscript{2+} currents at +20 mV increased by \(-83\%\). When we enhanced the amount of α1A-WT cRNA further by 11-fold (coinjection ratio of 1:10), the expression of Cav2.1 channels did not increase accordingly but was still 57\% higher than the control condition. This apparent reversal of the trend of increased expression of Cav2.1 channels at a 1:10 coinjection ratio, which is equivalent to doubling the amount of α1A-WT cRNA in the standard cRNA cocktail, was concurrent with the presence of the deterioration of the viability of *Xenopus* oocytes as a result the presumable Ca\textsuperscript{2+}-mediated cytotoxic effects mentioned above and thus should not be attributed to a suppression of the expression of Cav2.1 channels. In contrast, when we coexpressed WT and mutant α1A-subunits in a 1:1 molar ratio using the low-cRNA cocktail, the suppression effect of EA2 mutations was still significant, ranging from 50 to 88\% (Fig. 7B). In fact, except for E1761K, the suppression effects of EA2 mutations using the low-cRNA cocktail were actually more prominent than those using the standard cRNA cocktail (compare Fig. 2B with 7B). Altogether, our results indicate that the decreased expression of human WT-Cav2.1 channels in the presence of the EA2 mutants is indeed conferred by the specific dominant-negative effects of the α1A- mutants.

**Enhancing the amount of auxiliary subunits partially reverses the dominant-negative effects of nonsense EA2 mutants.** It is known that one of the major roles of Ca\textsuperscript{2+}--channel auxiliary subunits is to facilitate membrane insertion of α1A--subunits, thereby ensuring proper functional expression of Cav2.1 channels (2, 50). Consequently, EA2 mutants may suppress the functional expression of their WT counterpart by competing for the availability of auxiliary αδ-- and β\textsubscript{4}--subunits. To test this hypothesis, we asked whether the suppression effect bestowed by the five EA2 mutants could be reversed by the presence of an excessive amount of auxiliary αδ-- and β\textsubscript{4}--subunits. As shown in Fig. 8A, with the three nonsense mutants, up to a fourfold increase of the auxiliary subunits resulted in a small (~20\%) reversal of the dominant-negative effects. This partial reversal of suppression effects is not likely due to direct upregulation of the expression of α1A-WT by the auxiliary αδ-- and β\textsubscript{4}--subunits, because the expression level of WT-Cav2.1 channels did not significantly alter with an up to eightfold increase of auxiliary subunits (data not shown). To the contrary, in the presence of additional auxiliary subunits, the two missense mutants showed no significant change in the suppression effects (Fig. 8B). Thus our results demonstrate that competition for the availability of auxiliary αδ-- and β\textsubscript{4}--subunits can partially account for the dominant-negative effects of nonsense but not missense mutants.

**Dominant-negative effect of R1281x is dramatically reduced for the short isoform of human α1A--subunits.** R1281x mutant predicts a truncation mutation with a premature stop at the S1 segment of homologous domain 3 (Fig. 1A). Our finding that R1281x displays potent dominant-negative effects (Fig. 4) is in direct contrast to the findings of a previous study showing that R1281x cRNA coinjection into *Xenopus* oocytes failed to exhibit significant suppression effects on the current amplitude of human WT-Cav2.1 channels (56). One difference in the methodology between the two studies involves different splice isoforms of human α1A--subunits. The α1A-subunit we have used in the present study is the so-called long isoform of

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### Fig. 7

**A** normalized mean Ba\textsuperscript{2+} current amplitudes recorded from *Xenopus* oocytes expressing different concentrations of α1A-WT cRNA. Low-cRNA cocktail, in which amount of cRNA for α1A-, αδ-, and β\textsubscript{4}--subunits was only one-sixth that used for the standard cRNA cocktail, was used as control group (1:0) for oocyte injections. Appropriate amount of additional α1A-WT cRNA was then added to low-cRNA cocktail to mimic miscellaneous coexpression paradigms of α1A-WT and α1A-mutants described in Figs. 4 and 5. The mean amplitudes of Ba\textsuperscript{2+} currents recorded from *Xenopus* oocytes expressing different concentrations of α1A-WT cRNA (1:0, 1:1, 1:3, 1:5, and 1:10) were significantly different (\(P < 0.05\); 1-way ANOVA). In particular, enhancing the amount of α1A-WT cRNA 6-fold (1:5) or 11-fold (1:10) resulted in mean Cav2.1 current amplitudes significantly larger (\(P < 0.05\); t-test) than those in the control group (1:0). Data were normalized to mean +20-mV Ba\textsuperscript{2+} current amplitude of WT-Cav2.1 channels (1:0) using procedure described in Fig. 2B. Number of oocytes measured for each coexpression paradigm is shown in parentheses. **B** equimolar coexpression of EA2 mutants with WT-Cav2.1 channels using low-cRNA cocktail. Mean Ba\textsuperscript{2+} current amplitudes in the presence of EA2 mutants were significantly smaller (\(P < 0.05\); t-test) than those expressing WT-Cav2.1 channels alone. Number of oocytes measured for each coexpression paradigm is shown in parentheses.
DISCUSSION

Both missense and nonsense EA2 mutants exhibit dominant-negative effects on human WT-Cav2.1 channels comprising the long α1A-subunit isoform. EA2 is an autosomal dominant neurological disorder associated with mutations in the pore-forming α1A-subunits of human Cav2.1 channels. Because the majority of EA2 mutations result in a loss-of-function phenotype, both haploinsufficiency and dominant-negative effects may contribute to the mechanism leading to the cerebellar dysfunctions observed in patients with EA2. In theory, both haploinsufficiency and dominant-negative effects may result in the functional dominance of a loss-of-function mutation. Haploinsufficiency-conferred dominance usually is not a direct result of the reduced gene expression level per se; instead, it typically arises from an ineffective feedback system for the regulatory mechanism to compensate the reduced expression as exemplified by many human diseases caused by null mutations of transcription factors (42, 54). On the other hand, dominant-negative effects involve the interference of the function of a normal protein by the mutant one. Interestingly, different mutations of the same gene may lead to either haploinsufficiency or dominant-negative effects (31, 43).

To the best of our knowledge, we have presented herein the first evidence of human Cav2.1 channels that EA2 mutants, including three nonsense mutations (R1281x, R1549x, R1669x) and two
mis sense mutations (F1406C, E1761K), display prominent dominant-negative effects. Our studies suggest that the intense suppression effects conferred by both the missense and nonsense EA2 mutants are well beyond the expectation of simple suppression effects conferred by both the missense and non-sense EA2 mutants, when introduced into rabbit or rat $\alpha_{1A}$-subunit isoforms. Our studies suggest that the missense mutations (F1406C, E1761K), display prominent dominant-negative effects on human WT-Cav2.1 channels comprising the short $\alpha_{1A}$-subunit isoform. That the suppression effect of EA2 mutants varies considerably between the two splice variants may constitute a plausible rationale for reconciling our findings with those of the two previous reports (25, 56) showing that EA2 mutants failed to exhibit detectable dominant-negative effects on rabbit WT-Cav2.1 channels comprising the short $\alpha_{1A}$-subunit isoform (see last subsection under RESULTS). Our conclusion that the short $\alpha_{1A}$-subunit isoform of R1281x exhibits a considerable suppression effect when cojected at a 1:10 molar ratio, however, contrasts with the observation by Wappl et al. (56) that a 2-fold excess of R1281x cojection into Xenopus oocytes failed to exhibit any detectable effect. One difference in the methodology between the two studies lies in the auxiliary $\beta$-subunit subtype. Wappl et al. used the $\beta_1$-subunit, whereas we chose the $\beta_2$-subunit. A recent report demonstrated that a rat EA2-like $\alpha_{1A}$-truncation mutation that also predicts a premature stop at the S1 segment of homologous domain 3 displayed marked dominant-negative effects on rat WT-Cav2.1 channels comprising $\beta_3$-subunits (37). Similarly, a rabbit 95-kDa, two-domain form of $\alpha_{1A}$-subunit also showed significant dominant-negative effects on rabbit WT-Cav2.1 channels comprising $\beta_3$-subunits (3). Thus whether the difference in $\beta$-subunit subtype could explain the discrepancy between the two studies remains to be clarified.

Differences among heterologous expression systems used by investigators at various laboratories may also contribute to the foregoing conflicting results. One important issue that needs to be addressed in future studies, therefore, is whether the dom-
inert-negative effects that we observed in oocytes also exist in humans. The dominant-negative effect of the rat EA2-like α1A-truncation mutant was present in both oocyte and COS-7 expression systems (37), and the suppression effect of the rabbit two-domain isoform was demonstrated in a mammalian cell line (3). Hence, our present results are unlikely to represent an oocyte-specific phenomenon.

Potential molecular mechanisms underlying the dominant-negative effects of EA2 mutants. What is the mechanism underlying the dominant-negative effects of the five EA2 mutants? At least two scenarios might account for a reduction of the macroscopic current amplitudes of Ca2+ channels: 1) altered biophysical properties and 2) defective biosynthetic processes. We have shown that the reduction of the current amplitude of WT-Cav2.1 currents in the presence of EA2 mutants is not due to an alteration of the voltage-dependent gating property of the channel (Fig. 3). Previous single-channel analyses revealed that the gating kinetics of an EA2 missense mutant were significantly different from those for the WT (56).

Still unknown, however, is whether this EA2 mutant possesses any dominant-negative effect. A nonfunctional two-domain isoform of rabbit α1P-subunit (N-type) was previously shown to display a dominant-negative effect on rabbit WT-Cav2.2 channels. When these channels were functionally examined at the single-channel level, the biophysical properties of WT-Cav2.2 channels were found to remain virtually the same in the absence or presence of the truncated construct (38). Therefore, whether single-channel properties such as gating kinetics and single-channel conductance of human WT-Cav2.1 channels may be modified in the presence of EA2 mutants is still an open question.

In addition to an alteration of biophysical property, the mechanism of dominant-negative effect may also be explained by a decreased number of normal Cav2.1 channels in the plasma membrane, i.e., a faulty biosynthesis of WT-Cav2.1 channels in the presence of EA2 mutants. The biosynthetic process of ion channels can be divided into two general steps (9): biogenesis (e.g., protein folding and coassembly of multiple subunits) and membrane trafficking (e.g., clustering and localization).

It is still not clear whether the truncated α1A-subunits encoded by the nonsense mutations R1281X, R1549X, and R1669X can properly be inserted into the plasma membrane. Depending on the location of premature termination codons within an mRNA, mRNA derived from nonsense mutations may trigger nonsense-mediated mRNA decay, thereby preventing the production of the encoded truncated proteins (33). Consequently, some nonsense EA2 mutants may not be expressed at significant levels as a result of this posttranscriptional mRNA quality control mechanism. Alternatively, granted that normal transcriptional and translational processes do take place, a misfolded protein such as truncated channels may fail to pass protein quality control mechanisms taking place in the endoplasmic reticulum (ER), resulting in defective membrane trafficking and increased protein degradation (10, 32). Similar to the dominant-negative effects of mutations in cardiac human ether-a-go-go-related gene K+ channels (12, 27, 63), through yet to be identified mechanisms, truncated EA2 mutants may cause α1A-WT to misfold, thereby preventing proper trafficking and increasing protein degradation of the WT-Cav2.1 channel. Furthermore, an accumulation of misfolded protein in the ER may trigger an ER-mediated translation inhibition mechanism known as the unfolded protein response (17). A recent study of the mechanism of the dominant-negative effect of a rat EA2-like α1A-subunit nonsense mutant suggests that the truncated construct may interact with the cognate full-length α1A-WT to activate an unfolded protein response, leading to the suppression of the translation of α1A-WT (37). More experiments are needed to determine whether the human EA2 truncation mutants that we have studied can also activate a similar unfolded protein response.

The auxiliary α2δ- and β3-subunits play an essential role in facilitating the membrane targeting of α1A-subunits (2, 50). Accordingly, EA2 mutants may suppress the functional expression of Cav2.1 channels by competing for the availability of auxiliary subunits. Increasing the amount of auxiliary α2δ- and β3-subunits fourfold resulted in a small reduction of the dominant-negative effects of the three truncation mutants, whereas the same treatment did not significantly affect the suppression effects of the two missense mutants (Fig. 8), suggesting that competition for the availability of auxiliary α2δ- and β3-subunits may contribute to the dominant-negative effects of nonsense EA2 mutants. Further studies are required to determine whether competition for other chaperone proteins may also be involved in the suppression effect of EA2 mutants.

Our observation that competition for auxiliary subunits can partially account for the dominant-negative effects of nonsense but not missense EA2 mutants implies that the dominant-negative effects conferred by missense and nonsense mutants may not necessarily share the same molecular mechanism. In fact, the missense mutants F1406C and E1761K may not necessarily encode misfolded proteins. The loss-of-function phenotype of F1406C may reflect a significant alteration of the biophysical property of the channel conferred by the mutation located at the outer pore region (the S5-S6 linker) of homologous domain 3 (Fig. 1A). Similarly, with E1761K, the mutation site corresponds to one of the four conserved glutamate residues within the pore that dictate the divalent cation selectivity of the channel (11, 61). In rabbit cardiac L-type Ca2+ channels, individual replacements of each of the four conserved glutamate residues with lysine resulted in functional channels that were significantly more permeable to monovalent cations (61). Although there is no evidence yet, we speculate that both F1406C and E1761K mutants are likely to form properly folded α1A-subunits that, upon coassembly with the auxiliary subunits, are targeted for the plasma membrane. Whether (or how) the translation inhibition mechanism is applicable to the dominant-negative effects of missense mutants requires further investigation. In addition, it is of great interest to determine whether other identified EA2 missense mutants also possess dominant-negative effects.

For missense (and perhaps nonsense) EA2 mutants that retain proper membrane-trafficking properties, their loss-of-function phenotypes imply that they behave like virtually silent channels. The dominant-negative effects of these EA2 mutants can then be explained by competition between WT-Cav2.1 and silent Cav2.1 channels for a limited number of channel slots in the membrane. In other words, in the presence of EA2 mutants, the whole cell Ca2+ current amplitude decreases significantly because considerably fewer functional Cav2.1 channels gain access to a common membrane-trafficking pathway that does not distinguish be-
between WT and functionally silent channels. Such a slot theory was recently put forward by Cao et al. (4), who elegantly demonstrated that human WT-Cav2.1 and mutant Cav2.1 channels could compete for specific channel type-prefering slots located at presynaptic terminals and that the mutant channels’ impairment in contributing to neurotransmission matched precisely their deficiency in supporting whole cell Ca$^{2+}$ current density. Notably, one of the mutants tested by Cao et al. involved a quadruple-mutation (E4A) of the four glutamate residues at the selectivity filter, similarly to the missense variant is the dominant isoform (44), quantitative and qualitative alterations in the expression pattern of Cav2.1 channels lead to dire consequences, such as erroneous wiring of synaptic output neurons of the cerebellar cortex, the foregoing neuro-pathological scenarios may in turn contribute to the development of EA2-related ataxic symptoms.

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