Biochemical and Biophysical Evidence for $\gamma_2$ Subunit Association with Neuronal Voltage-activated $\text{Ca}^{2+}$ Channels*

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A novel gene (Caeng2; $\gamma_2$) encoding a protein similar to the voltage-activated $\text{Ca}^{2+}$ channel $\gamma_1$ subunit was identified as the defective gene in the epileptic and ataxic mouse, stargazer. In this study, we analyzed the association of this novel neuronal $\gamma_2$ subunit with $\text{Ca}^{2+}$ channels of rabbit brain, and the function of the $\gamma_2$ subunit in recombinant neuronal $\text{Ca}^{2+}$ channels expressed in Xenopus oocytes. Our results showed that the $\gamma_2$ subunit and a closely related protein (called $\gamma_3$) co-sedimented and co-immunoprecipitated with neuronal $\text{Ca}^{2+}$ channel subunits in vivo. Electrophysiological analyses showed that $\gamma_2$ co-expression caused a significant decrease in the current amplitude of both $\alpha_1(B_2,2.2)$-class (36.8%) and $\alpha_1(A_{2.1})$-class (39.7%) $\text{Ca}^{2+}$ channels ($\alpha_2\beta_2\delta\gamma_2\gamma_3$). Interestingly, the inhibitory effects of the $\gamma_2$ subunit on current amplitude were dependent on the co-expression of the $\alpha_2\delta$ subunit. In addition, co-expression of $\gamma_2$ or $\gamma_1$ also significantly decelerates the activation kinetics of $\alpha_1\beta\gamma_2$-class $\text{Ca}^{2+}$ channels. Taken together, these results suggest that the $\gamma_2$ subunit is an important constituent of the neuronal $\text{Ca}^{2+}$ channel complex and that it down-regulates neuronal $\text{Ca}^{2+}$ channel activity. Furthermore, the $\gamma_2$ subunit likely contributes to the fine-tuning of neuronal $\text{Ca}^{2+}$ channels by counterbalancing the effects of the $\alpha_2\delta$ subunit.

Voltage-activated $\text{Ca}^{2+}$ channels play a major role in many fundamental physiological processes including neurotransmission, muscle contraction, intracellular signaling, hormone secretion, and development. Understanding the molecular regulation of these channels is critical for the comprehension of these major physiological phenomena. The high voltage-activated $\text{Ca}^{2+}$ channel (called $\text{Ca}^{2+}$ channel hereafter) consists of at least three subunits: a main subunit, $\alpha_1$, and two auxiliary subunits, $\beta$ and $\alpha_2\delta$ (1, 2). An additional auxiliary subunit, $\gamma$, initially detected only in skeletal muscle, has been recently suggested to be a component of the neuronal $\text{Ca}^{2+}$ channel complex (3). The regulation of $\text{Ca}^{2+}$ channels by the $\beta$ and $\alpha_2\delta$ subunits has been extensively studied. Both $\beta$ and $\alpha_2\delta$ subunits have been found to increase specific ligand binding and to modulate electrophysiological properties of $\text{Ca}^{2+}$ channels including current density, voltage dependence, and current kinetics (2). However, much less is known about the function of the skeletal muscle $\gamma$ subunit ($\gamma_1$). Some controversial results of $\gamma_1$ effects on current amplitude, voltage dependence, current kinetics, or toxin binding have been observed (4–9).

A molecular genetic study of the stargazer mouse revealed a novel gene responsible for absence epilepsy and ataxia in this animal model. The study suggested that this novel gene encodes a $\gamma$ subunit for neuronal $\text{Ca}^{2+}$ channels that has been named $\gamma_2$ (or stargazin). The first 200 amino acids of the $\gamma_2$ and $\gamma_1$ sequence share 25% identity and 39% similarity. The exon-intron organization and predicted secondary structure of the $\gamma_2$ subunit are very similar to that of the $\gamma_1$ subunit (3). Previous biophysical studies using recombinant $\text{Ca}^{2+}$ channels have indicated an inhibitory role for the $\gamma_2$ subunit based on changes in the voltage dependence of steady-state inactivation of the channels (3, 10). In addition, a recent finding of novel $\gamma$ isoforms ($\gamma_2$, $\gamma_4$, $\gamma_5$) suggests a possibility that a $\gamma$ gene family originated through tandem and chromosome duplication (11). On the other hand, a recent study has suggested a role of the $\gamma_2$ subunit on the trafficking/clustering of AMPA receptor 7 receptors (12).

Mutations in the $\text{Ca}^{2+}$ channel subunits have been implicated in the etiology of absence epilepsy and ataxia in mice: four $\alpha_1A$ mutations in the tottering, leaner, rolling, and rocker mice (13–15); a $\beta_4$ mutation in the lethargic mouse (16); a $\alpha_2\delta-2$ mutation in the ducky mouse (17); and a $\gamma_2$ mutation in the stargazer mouse (3). Understanding $\text{Ca}^{2+}$ channel regulation at the molecular level will lead to further comprehension of the mechanisms underlying these neurological disorders.

In this study, by showing the association of the $\gamma_2$ subunit with other $\text{Ca}^{2+}$ channel subunits, we provide biochemical data supporting the hypothesis that the $\gamma_2$ subunit is a component of the neuronal $\text{Ca}^{2+}$ channel complex. Furthermore, by examining the function of the $\gamma_2$ subunit using recombinant $\alpha_1\beta\gamma_2$ and $\alpha_1\Delta\gamma_2$-class $\text{Ca}^{2+}$ channels expressed in Xenopus oocytes, we show that the novel $\gamma_2$ subunit participates in the modulation of the neuronal $\text{Ca}^{2+}$ channels. Our results demonstrate that the $\gamma_2$ subunit is a part of the neuronal $\text{Ca}^{2+}$ channel complex and has an $\alpha_2\delta$-dependent inhibitory effect on the channel activity.

EXPERIMENTAL PROCEDURES

Partial Purification of Neuronal $\text{Ca}^{2+}$ Channels—Brain microsomes were prepared from rabbit and mouse as described previously (18). From the microsomes (200 mg of rabbit and 50 mg of mouse micro-

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1 The abbreviations used are: AMPA, $\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid; WGA, wheat germ agglutinin; MES, 4-morpholineethanesulfonic acid.
FIG. 1. Association of the γ2 and γ3 subunits with other neuronal Ca2+ channel subunits. A, sucrose gradient fractionation of neuronal Ca2+ channels. The numbers at the top indicate the fraction of the sucrose gradient from top to bottom. Equal volume of proteins (80 μl) were loaded in each lane. B, characterization of the protein bands recognized by the γ2 or γ3 antibodies. The protein samples (100 μg each) were from the stargazer mice (stg) or their wild-type littermates (WT). The antibodies used for the Western blot analyses are indicated at the bottom. C, co-immunoprecipitation of the γ2 and γ3 subunits by antibodies specific for neuronal Ca2+ channel subunits. The first lane (Input) was loaded with the protein aliquot saved before immunoprecipitation. Sheep 37 (anti-α1, VD2, anti-β), and Rabbit 239 (anti-γ2), specific for the Ca2+ channel subunits, α1, β, and γ2, respectively, were used for immunoprecipitation. Equal amounts of proteins (50 μg) were loaded in each lane. The antibodies used for immunoprecipitation are indicated at the top of the other lanes. Molecular mass standards (×10−3) are indicated on the left. Data are representative of at least three independent experiments.

Interactions between γ2 and Neuronal Ca2+ Channels

Some of the Ca2+ channel complexes were extracted with solubilization buffer containing (in mM) 50 Tris-HCl, pH 7.4, 500 NaCl, a mixture of protease inhibitors, and 1% digitonin (Biochimica & Synthetica, Stad, Switzerland) by rotating end-over-end at 4°C for 1 h. After centrifugation at 142,413 × g for 37 min, solubilized proteins in the supernatant were then mixed with wheat germ agglutinin (WGA)-agarose beads (Vector Laboratories, Burlingame, CA) and rotated end-over-end at 4°C overnight. After washing three times with three bed volumes of ice-cold wash buffer (Buffer I containing (in mM) 50 Tris-HCl, pH7.4, 500 NaCl, a mixture of protease inhibitors, and 0.1% digitonin), WGA-bound proteins were eluted with elution buffer (Buffer I + 0.3 M N-acetyl-D-glucosamine (Sigma)). The WGA eluant was concentrated to 0.5 ml in an Ultrafree-15 centrifugal filter device (Millipore, Bedford, MA), and applied to a 5–30% sucrose gradient density buffer (Buffer I + 5–30% sucrose). The gradients were centrifuged at 215,000 × g for 90 min. Fractions (0.8 ml) were collected from the top of the gradients using a density gradient fractionator (Auto Densi-flow, Labconco, Kansas City, MO).

Antibodies—Polyclonal antibodies, Sheep 37, Sheep 46, Rabbit 136, Sheep 49, Rabbit 145, and Rabbit 239, specific for the Ca2+ channel subunits, α1, β, and γ2, respectively, have been described previously (3, 19–22). The γ2 subunit-specific polyclonal antibody, Rabbit 302, was generated by Genemed Synthesis (San Francisco, CA) against an amino-terminal cysteine 12-mer peptide (Research Genetics, Huntsville, AL) corresponding to residues 273–281 of mouse γ2 subunit primary structure. Monoclonal antibody VD2, which recognizes all Ca2+-channel β subunits, has been described previously (23).

Immunoprecipitation of Ca2+ Channel Subunits—Antibodies were cross-linked to protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) by using dimethyl pimelimidate as described elsewhere (24). The partially purified neuronal Ca2+ channel complexes were incubated with the antibody-protein A-agarose beads at 4°C overnight on a rolling platform. After washing the beads three times with three bed volumes of ice-cold wash buffer (Buffer I), Ca2+ channel subunits bound to the antibodies were eluted with 50 mM glycine-HCl, pH 2.5, and the pH was immediately neutralized with 0.1 volume of 1 M Tris-HCl, pH 8.0.

Western Blot Analysis—The WGA eluants, sucrose gradient fractions, or immunoprecipitated proteins were resolved in 4–15% gradient SDS-polyacrylamide gel electrophoresis under reducing condition (2% β-mercaptoethanol) and transferred to polyvinylidene difluoride membrane (Millipore). The polyvinylidene difluoride blots were cut and probed separately with affinity-purified antibodies against the subunits of the Ca2+ channel complex or Na+/K+ ATPase at 4°C overnight. After staining with Horse radish peroxidase-conjugated secondary antibodies (Roche Molecular Biochemicals), the blots were developed by enhanced chemiluminescence (ECL) (SuperSignal™ or DuraSignal™, Pierce, Rockford, IL), and imaged using an image capturing system (MultiImage™, Alpha Innotech, San Leandro, CA).

cDNA Clones—cDNA clones used were as follows: rabbit brain α1A cDNA (4), rat brain α1H cDNA (25), rat brain β3 (GenBank™ accession no. MS7651), rat brain α2β2 (GenBank™ accession no. MS8621), rabbit skeletal γ1 (26), and mouse brain γ2 (3). As expression vectors, pcDNA3 (Invitrogen, Carlsbad, CA) was used for α1H, γ1, and γ2; pSP72 (Promega, Madison, WI) for α1H; pBSTA (27) for α1H; and pGEM3 (Promega) for β3.

Preparation of Xenopus Oocytes—Female Xenopus laevis were purchased from Nasco (Fort Atkinson, WI). Fros were maintained under a 12-h light/12-h dark cycle at 18°C. Ovarian lobes were surgically removed from frogs that had been anesthetized by hypothermia. To facilitate injection and recording, the follicle cell layer was enzymatically digested with 2 ml/mg collagenase type I (Sigma) for 70 min in Ca2+-free OR-2 solution containing (in mM) 82.5 NaCl, 2.5 KCl, 1 MgCl2, and 5 HEPES-NaOH, pH 7.6. Oocytes at stages V and VI were selected and washed several times with Ca2+-free OR-2 solution and subsequently
placed in ND96 solution containing (in mM) 96 NaCl, 15 KCl, 1 MgCl2,
1.8 CaCl2, 2.5 sodium pyruvate, and 5 HEPES-NaOH, pH 7.6, plus 1%
penicillin/streptomycin. Before cRNA injection, the oocytes were incu-
bated overnight at 18 °C in ND96 solution.

Heterologous Expression of Ca2+ Channel Subunits—Linearized plasmids were in vitro transcribed with T7 (or SP6 for α1) polymerase
transcription kits (mMESSAGE mACHINE, Ambion, Austin, TX).
Transcribed cRNA was purified using the RNaseasy kit (Qiagen, Stan-
ford, CA), and then analyzed by gel electrophoresis and stored at
−20 °C. Oocytes were injected with 46 nl of various cRNA mixtures of
Ca2+ channel subunits using a nano-injector (Drummond, Broomall,
PA) at the following ratio: 2:2:1 = α1:α1β3ε;γ. The amount of cRNA
injected was varied in each experiment to get current amplitudes under
optimal voltage control. Injected oocytes were incubated at 18 °C in
ND96 solution for 4–5 days before electrophysiological recording.

Electrophysiological Recordings and Data Analyses—Ba2+ currents through Ca2+ channels were recorded by the two-electrode voltage-
clamp technique (28) with a TEV-200 amplifier (Dagan, Minneapolis,
MN) at room temperature. Microelectrodes were pulled from borosili-
cate glass capillary (Kimble Glass Co., Vienland, NJ) using a horizontal
puller (Sutter Instrument, Novato, CA). Both voltage and current elec-
trodes were filled with 3 M KCl and had initial tip resistances of 0.5–1.0
megohm. The recording chamber (500-μl volume) was filled with re-
cord solution containing (in mM) 10 BaCl2(OH)2, 2 KCl, 0.1 EGTA, 80
NaOH, 1 niflumic acid, and 10 HEPES-MES, pH 7.2. Ca2+-activated
Cl− outward currents were minimized by the use of Ba2+ as charge
carrier, low concentration of Cl− in the solutions, and niflumic acid (a
Cl− current blocker). Unless otherwise indicated, test potentials were
applied for 2 s from a holding potential of −90 mV using pClamp 6
software (Axon Instruments, Foster City, CA). Output signals were
filtered at 1 kHz and sampled at 5 kHz. Data were digitized with a TL-1
interface (Axon Instruments), and the results were analyzed by pClamp
6 and SigmaPlot 4.01 (SPSS Inc., Chicago, IL). Leak and capacitance
currents were subtracted on-line by a P/6 protocol. If present, residual
capacitance was blanked.

I-V curves were fitted using a modified Boltzmann equation of the form:
I = I_{m}\exp\left(-\frac{V - \text{V}_{1/2}}{k}\right) / (1 + \exp\left(-\frac{V - \text{V}_{1/2}}{k}\right)),
where I represents current amplitude, \(G_{\text{m}}\) maximum conductance, \(V_m\) test potential, \(E\) reversal potential, \(V_{1/2}\) potential of half-activation, and \(k\) slope factor.

Steady-state activation curves were also described by a modified Bolt-
zmann equation: \(G = G_{\text{m}}/\left(1 + \exp\left(V_m - V_{1/2}\right)/k\right)\),
where \(G\) represents conductance obtained from the equation:
\(G = I(V_m - E)\). Steady-state inactivation curves were also described by a modified Boltzmann equation:
\(I = I_{m}\exp\left(-\frac{V - \text{V}_{1/2}}{k}\right) / (1 + \exp\left(V - \text{V}_{1/2}\right)/k)\). To obtain the estimates of the
activation and inactivation rates, data were fit to single- and
two-exponential equations, respectively, using the fitting routines
of pClamp 6 software.

Statistical Analysis—Each experiment was repeated at least three
times using different batches of oocytes or brain from different animals.
Data are presented as means ± S.E. of the mean, and the number of

 FIG. 2. Regulation of current amplitude of neuronal Ca2+ channels by the γ3 subunit. A, superimposed current traces of α1γγ-class channels. B, current-

voltage (I-V) relationships of α1γγ-class channels. C, superimposed current traces of α1γγ-class channels. D, I-V relationships of α1γγ-class channels. Current traces were averaged from three representative cells in each group of oocytes, and the voltage protocol is shown above the traces (A and

C). Ca2+ channel subunit composition is listed in the inset, and the number of re-
corded cells is indicated in parentheses (B and D). Data are representative of at least three independent experiments.

 FIG. 3. Modulation of voltage dependence of neuronal Ca2+ channels by the γ3 subunit. A & B, Superimposed plots of steady-

state activation (G/G_{\text{m}}) and inactivation (I/I_{\text{m}}) curves from α1γγ-class (A) and α1γγ-class (B) channels. Ca2+ channel subunit composition is listed in

the inset, and the number of recorded cells is indicated in parentheses. Data are representative of at least three independent

experiments.

oocytes is indicated in the figures and Table I. Data were analyzed by two-way analysis of variance. When significant F-values were encountered,
the different treatments were compared using the Tukey multiple comparisons test. Probability (p) of 0.05 or less was considered
TABLE I

Kinetic parameters of the \( \alpha_{11}\alpha \) and \( \alpha_{1A}\) class \( Ca^{2+} \) channels

<table>
<thead>
<tr>
<th>Properties</th>
<th>( \alpha_{11}\alpha_{3}\beta )</th>
<th>( \alpha_{11}\alpha_{2}\gamma )</th>
<th>( \alpha_{1A}\beta_{3}\gamma_{2} )</th>
<th>( \alpha_{1A}\beta_{2}\delta \gamma_{2} )</th>
<th>( \alpha_{1A}\beta_{2}\gamma_{2} )</th>
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<tbody>
<tr>
<td>Steady-state activation parameters</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>( V_{1/2} ) (mV)</td>
<td>-12.10 ± 0.69**</td>
<td>-5.63 ± 0.87</td>
<td>-9.42 ± 0.36**</td>
<td>-7.54 ± 0.59</td>
<td>-4.35 ± 2.03</td>
</tr>
<tr>
<td>( k ) (mV)</td>
<td>-4.61 ± 1.3*</td>
<td>-5.59 ± 0.29</td>
<td>-5.36 ± 0.12</td>
<td>-5.68 ± 0.18</td>
<td>-5.42 ± 0.19</td>
</tr>
<tr>
<td>( n )</td>
<td>5</td>
<td>9</td>
<td>8</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Steady-state inactivation parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{1/2} ) (mV)</td>
<td>-43.34 ± 1.26</td>
<td>-38.86 ± 0.81</td>
<td>-42.44 ± 1.73</td>
<td>-41.95 ± 0.89</td>
<td>-26.52 ± 6.51</td>
</tr>
<tr>
<td>( k ) (mV)</td>
<td>10.13 ± 0.55</td>
<td>9.51 ± 0.33</td>
<td>9.92 ± 0.76</td>
<td>10.22 ± 0.36</td>
<td>6.51 ± 0.34</td>
</tr>
<tr>
<td>( n )</td>
<td>6</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>11</td>
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</table>

The statistical analysis was performed using SYSTAT 7.0 (SPSS Inc.).

RESULTS

Association of the \( \gamma_{2} \) Subunit with Neuronal \( Ca^{2+} \) Channel Subunits—The association of the \( \gamma_{2} \) subunit with the neuronal \( Ca^{2+} \) channel complex was investigated through sucrose gradient fractionation analysis as follows. \( Ca^{2+} \) channel complexes were partially purified from the rabbit cerebellum as described under “Experimental Procedures.” Western blot analysis of sucrose gradient fractions with anti-\( \alpha_{1B} \), anti-\( \alpha_{2} \), anti-\( \beta_{2} \), anti-\( \beta_{3} \), and anti-\( \gamma_{253} \) antibodies showed that the \( \alpha_{1B}, \alpha_{2}, \beta_{2}, \beta_{3}, \) and \( \gamma_{253} \) co-sedimented in fractions 9–12 (Fig. 1A). Considering the much smaller molecular masses of the \( \gamma_{253} \) (34 and 38-kDa), \( \beta_{3} \) (57-kDa) or \( \beta_{4} \) (57-kDa) subunits compared with that of the \( \alpha_{1B} \)
Interactions between \( \gamma_2 \) and Neuronal \( Ca^{2+} \) Channels

To confirm that the co-sedimentation of the \( \gamma_2 \) and \( \gamma_3 \) subunits with the \( \alpha_{1B} \) subunits was due to a specific interaction with the \( Ca^{2+} \) channel complex, sucrose gradient fractions (fraction 9–12) containing both \( \alpha_{1B} \) and \( \gamma_2 \) subunits (Fig. 1A) were pooled and subjected to immunoprecipitation analyses as described under “Experimental Procedures.” Polyclonal anti-\( \alpha_1 \) antibody recognizing \( \alpha_{1A/B} \) subunits of \( Ca^{2+} \) channel immunoprecipitated both the \( \gamma_2 \) and \( \gamma_3 \) subunits (Fig. 1C, second lane). Monoclonal anti-\( \beta \) antibody, which recognizes all \( Ca^{2+} \) channel \( \beta \) subunits, was also able to immunoprecipitate the \( \gamma_2 \) and \( \gamma_3 \) subunits (Fig. 1C, third lane). The same blots were probed with the anti-\( \alpha_2 \) antibodies to show that the whole neuronal \( Ca^{2+} \) channel complexes were immunoprecipitated. Furthermore, the \( \alpha_2 \) band (140 kDa) was detected by immunoprecipitation with the anti-\( \gamma_2/3 \) antibody (Fig. 1C, fourth lane). In addition, to rule out the possibility of nonspecific precipitation by these antibodies, the same blot was probed with an anti-\( Na^+/K^+ \) ATPase antibody (Affinity Bioreagents, Inc., Golden, CO). The \( Na^+/K^+ \) ATPase was detected in the pooled fraction of sucrose gradient prior to immunoprecipitation (Fig. 1C, first lane, Input), but was not immunoprecipitated with any of the calcium channel subunit antibodies (Fig. 1C, second through fourth lanes). These immunoprecipitation results strongly suggest that the \( \gamma_2 \) and \( \gamma_3 \) subunits bind to neuronal \( Ca^{2+} \) channel complexes composed of \( \alpha_{1A/B} \), \( \alpha_2 \), and \( \beta \) subunits.

Taken together, the co-sedimentation and co-immunoprecipitation of the \( \gamma_2 \) and \( \gamma_3 \) subunits with other neuronal \( Ca^{2+} \) channel subunits constitute what is to our knowledge the first biochemical evidence that the \( \gamma_2 \) and \( \gamma_3 \) subunits are structural components of neuronal \( Ca^{2+} \) channel complexes of the \( \alpha_{1B} \)- and \( \alpha_{1A} \)-class.

**Effect of the \( \gamma_2 \) Subunit on the Current Amplitude of Recombinant \( \alpha_{1B} \) and \( \alpha_{1A} \)-Class \( Ca^{2+} \) Channels**—Having shown that the \( \gamma_2 \) subunit is an important part of the neuronal \( Ca^{2+} \) channels, we next explored the effects of this subunit on \( Ba^{2+} \) current through \( \alpha_{1B}\beta_{3}\alpha_2 \) channels using *Xenopus* oocytes as an expression system. Fig. 2A shows representative current traces from four groups of oocytes injected with different subunit compositions. Co-expression of \( \gamma_2 \) or \( \gamma_3 \) significantly decreased current amplitude. This effect was consistently observed at most potentials examined, as shown in Fig. 2B. In the representative experiments shown in Fig. 2A and B, peak current amplitude was \(-1.22 \pm 0.08 \mu A (n = 9) \) in cells expressing \( \alpha_{1B}\beta_3\alpha_2\delta \) and significantly decreased to \(-0.76 \pm 0.07 \mu A (n = 8) \) and \(-0.82 \pm 0.08 \mu A (n = 9) \) upon co-expression of \( \gamma_2 \) (\( p < 0.001 \)) and \( \gamma_3 \) (\( p < 0.003 \)), respectively. Overall, the cells co-expressing \( \gamma_2 \) (n = 31) showed 36.8 \% decrease of peak current amplitude compared with the cells expressing only \( \alpha_{1B}\beta_3\alpha_2\delta \) (n = 25). A similar effect of the \( \gamma_2 \) subunit on current amplitude was observed in oocytes expressing \( \alpha_{1A} \)-class \( Ca^{2+} \) channels (Fig. 2, C and D). Fig. 2C shows the current traces from two groups of oocytes in the absence and presence of \( \gamma_3 \). In this case, peak amplitude decreased significantly from \(-0.40 \pm 0.04 \mu A (n = 11) \) to \(-0.18 \pm 0.03 \mu A (n = 5) \) in cells expressing \( \alpha_{1A}\beta_3\alpha_2\delta \) and \( \alpha_{1A}\beta_3\delta \gamma_2 \) (\( p < 0.007 \)), respectively. This effect was consistently observed in the \( \pm 40 \) mV range (Fig. 2D). Overall, the cells co-expressing \( \gamma_2 \) (n = 36) showed 39.7 \% decrease of peak current amplitude compared with the cells expressing only \( \alpha_{1A}\beta_3\alpha_2\delta \) (n = 25).

**Effect of the \( \gamma_2 \) Subunit on Voltage Dependence of \( \alpha_{1B} \) and \( \alpha_{1A} \)-Class \( Ca^{2+} \) Channels**—Since it could be possible that the effect of the \( \gamma \) subunits on current amplitude is due to a change...
in the voltage dependence of steady-state activation and/or inactivation, we next studied the voltage-dependent properties of activation and inactivation of α1β2- and α1A-class Ca2+ channels. Fig. 3 (A and B) shows the steady-state activation of the recombinant Ca2+ channels plotted as a function of the test potential. Data were fitted with a modified Boltzmann equation to calculate potentials for half-maximal activation and slope factors, and the results are summarized in Table I. Although there was no significant shift of the membrane potential for half-maximal activation in both classes of channels by γ2 co-expression, the slope factor of the activation curve for α1β2δβ2δ channel was affected by γ2 co-expression. In addition, the properties of voltage-dependent inactivation were studied by applying 2.0-s pre-pulses ranging successively from −100 to 50 mV in 10-mV voltage steps, followed by a 0.5-s step depolarization to 0 mV. The averaged data were plotted as a function of voltage and fitted with a modified Boltzmann equation as shown in Fig. 3 (A and B), and potentials for half-maximal inactivation and slope factors are summarized in Table I. Although γ2 co-expression did not affect significantly the membrane potential for half-maximal inactivation of the α1β2δβ2δ channel, the slope factor of the steady state inactivation curve for α1β2δβ2δ channel was affected by the co-expression of the γ2 subunit (Table I).

Effect of the γ2 Subunit on Kinetics of α1β2- and α1A-class Ca2+ Channels—The inactivation kinetics of the α1M- and α1A-class channels was assayed by applying a 2.0-s pulse from a −90 mV holding potential to various membrane potentials. Inactivation time constants were then obtained by fitting the decaying phase of the currents to a two-exponential equation. In Fig. 4 (A and B), these time constants are plotted as function of the test potential. The properties of inactivation kinetics were similar in both α1M- and α1A-class channels in terms of inactivation rate and the voltage dependence of inactivation rate. Fast inactivation time constants (τi) in both types of channels were around 100 ms in a wide range of test potentials. In contrast, the voltage dependence of slow inactivation in both α1M- and α1A-class channels showed a U-shape. Similar U-shaped voltage dependence of inactivation rate of α1M-class channels was reported previously (29). Slow inactivation time constants (τs) were highest at −20 mV and decreased to about 400 ms around 10 mV, and then increased again at voltages above 30 mV. Overall, inactivation kinetics of neither α1M- nor α1A-class Ca2+ channels was modified by γ2 co-expression (Fig. 4, A and B). For example, at 10 mV (peak current potential), τs values were 61.80 ± 1.85 ms (n = 7) and 62.33 ± 2.33 ms (n = 6), and τi 473.20 ± 19.09 ms (n = 7) and 469.00 ± 21.31 ms (n = 6) in α1Mβ2δβ2δ and α1Mβ2δβ2γ2 channels, respectively (Table I).

Similarly, the effects of the γ2 subunit on activation kinetics were analyzed by comparing the activation time constant (τact) in a series of test potentials (Fig. 4C) ranging from −20 to +30 mV. Time constants were measured by fitting the rising phase of current to a single-exponential equation. As illustrated in Fig. 4C, the activation of currents was significantly decelerated by γ2 co-expression at negative potentials (see also Table I). The differences in activation at a test potential of −10 mV are depicted in Fig. 4D using representative normalized current traces from a group of oocytes expressing α1M-class channels in the absence and presence of the γ subunits. These traces clearly illustrate that the rising phase of the currents in the presence of the γ subunits activate slower than the currents recorded in oocytes expressing only α1Mβ2δβ2δ. Consistent with this, at −10 mV τact values were 2.43 ± 0.07 ms (n = 9) and 2.98 ± 0.11 ms (n = 8) in α1Mβ2δβ2δ and α1Mβ2δβ2γ2 channels, respectively (p < 0.014). The activation kinetics of α1A-class channel was analyzed similarly; however, in this case γ2 co-expression did not change activation kinetics (data not shown).

The Inhibitory Effect of the γ2 Subunit Is Dependent on the Co-expression of the α2δ Subunit—To investigate the mechanism of the γ2 inhibitory effect on current amplitude, we analyzed the peak currents through α1A-class Ca2+ channel in various combinations of auxiliary subunits in the presence or absence of the γ2 subunit (Fig. 5A). The amount of cRNA injected for the experiment presented in Fig. 5A was much higher than that of the other experiments to obtain a better expression of α1A subunits without other auxiliary subunits. The current amplitude of α1A channels was not significantly modified by the γ2 co-expression; current amplitude of α1A and α1Aγ2 channels were −37 ± 2.2 nA (n = 6) and −33 ± 2.8 nA (n = 6). In contrast, γ2 co-expression decreased the current amplitude of α1A channels when the α2δ subunit was present. The current amplitude decreased from −99 ± 1.1 nA (n = 13) in α1Aα2δ channels to −37 ± 1.1 nA (n = 6) in α1Aα2δγ2 channels (p < 0.018). Interestingly, the current amplitude of α1Aβ3 channel was not affected by γ2 co-expression; the current amplitude of α1Aα2δ β3 channel was −560 ± 42 nA (n = 6) and −570 ± 40 nA (n = 12), respectively. However, in the α1Aβ3δβ2δ channels, the current amplitude significantly decreased from −6111 ± 270 nA (n = 8) to −4357 ± 328 nA (n = 6) by γ2 co-expression (p < 0.02).

Similarly, a current amplitude analysis performed in the α1B-class Ca2+ channels also showed a significant inhibitory effect by the γ2 subunit only when α2δ was co-expressed (Fig. 5B). The current amplitude of α1Bβ3 channels was not affected by γ2 co-expression; the current amplitude of α1Bα2δ and α1Bβ3γ2 channels was −141 ± 9.1 nA (n = 14) and −123 ± 11 nA (n = 8), respectively. On the other hand, in the α1Bβ3δβ2δ channels, the current amplitude significantly decreased from −900 ± 73 nA (n = 12) to −613 ± 48 nA (n = 8) by γ2 co-expression (p < 0.02). Therefore, it seems that the inhibitory effect of the γ2 subunits on neuronal Ca2+ channel current amplitude is dependent on the α2δ co-expression.

DISCUSSION

Interaction of the γ2 Subunit with the Neuronal Ca2+ Channel Complex—In this study, the association of the γ2 subunit with neuronal Ca2+ channel complexes was analyzed through sucrose density gradient fractionation and immunoprecipitation. Both biochemical analyses consistently demonstrated that the γ2 subunits are associated with neuronal Ca2+ channels (α1M- and α1A-class). In addition, the biochemical analyses in this study also showed, for the first time, that the protein expression of the γ2 subunit is totally absent in the brain of the stargazer mouse and that the γ2 subunit is also associated with the neuronal Ca2+ channels.

Our biochemical and biophysical data strongly suggest that γ2 is an auxiliary subunit of neuronal Ca2+ channels like the β and αδ subunits. As shown in Fig. 1, the γ2 association with neuronal Ca2+ channels was confirmed by both co-sedimentation and co-immunoprecipitation of the γ2 subunit with the other components of neuronal Ca2+ channels. Additionally, our biophysical studies suggest an inhibitory role for the γ2 subunit on neuronal Ca2+ channel activity. This is consistent with a recent patch clamp study in brain slices of the stargazer mouse that showed the current amplitude of voltage-activated Ca2+ channel in thalamocortical relay neurons is significantly increased (30). Taken together, these data strongly suggest that the γ2 subunit is a component of neuronal Ca2+ channels in vivo.

Interestingly, another recent study by Chen et al. (12) suggested that the γ2 subunit is involved in the regulation of synaptic targeting/clustering of AMPA receptors. This raises an intriguing possibility that the γ2 subunit is involved not only in Ca2+ current modulation but also in AMPA receptor traf-
ficking. However, Chen et al. showed no significant change of the Ca$^{2+}$ channel current in the isolated cerebellar granule cells between the stargazer and wild-type mice. One possible explanation for the unaltered Ca$^{2+}$ channel current could be the compensation for the loss of $\gamma_2$ by other $\gamma$ isoforms. Consistent with this possibility, five $\gamma$ isoforms have been reported (3, 10, 11, 26, 31). Furthermore, it has been demonstrated that the loss of $\beta_1$ subunit did not cause any significant change in the Ca$^{2+}$ channel current of Purkinje neurons from lethargic mouse, due to increased steady-state association of $\alpha_1$ subunit with the remaining $\beta_2$-isoforms (32). Similar functional compensation might occur in the Ca$^{2+}$ channels of the cerebellar granule cells of the stargazer mouse.

In the sucrose gradient analysis, the $\alpha_2\delta$ subunit predominantly sedimented in fractions 9–12, where all other subunits sedimented. However, a peak of $\alpha_2\delta$ that dissociates from the Ca$^{2+}$ channel complex during the purification process.

The Modulation of Neuronal Ca$^{2+}$ Channels by the $\gamma_2$ Subunit—The electrophysiological analysis in this study brought out two major points about the modulation of neuronal Ca$^{2+}$ channels by the $\gamma_2$ subunit. First, the main function of the $\gamma_2$ subunit on neuronal Ca$^{2+}$ channels seems to be an inhibitory effect on functional activity of these channels. There were strong and consistent inhibitory effects of $\gamma_2$ on current amplitude (37–40%) through all of our experiments (Figs. 2 and 5). The activation kinetics of the $\alpha_{1B}$-class Ca$^{2+}$ channel was also significantly decelerated by $\gamma_2$ co-expression (Fig. 4, C and D). Consistent with our finding, the patch clamp studies of the stargazer mouse showed 40% decrease of current amplitude of high voltage-activated Ca$^{2+}$ channels (30). Moreover, recent studies of $\gamma_1$ null mice reported an increase of current amplitude of Ca$^{2+}$ channel as the main alteration after $\gamma_1$ gene ablation in skeletal muscle (33, 34). In addition, the consistent $\gamma_2$ inhibitory effect in vitro and in vivo suggests that the down-regulation of neuronal Ca$^{2+}$ channel activity by the $\gamma_2$ subunit may be important for the prevention of neuronal hyperexcitability that has been suggested as a mechanism of epilepsy in the stargazer mouse.

Second, our results suggest that the inhibitory effect of $\gamma_2$ on current amplitude depends on the co-expression of the $\alpha_2\delta$ subunit. The experimental results in Figs. 2 and 4 suggest that $\gamma_2$ might counteract the $\alpha_2\delta$ effects on neuronal Ca$^{2+}$ channels. The increase in current amplitude induced by $\alpha_2\delta$ was reversed by $\gamma_2$ co-expression (Fig. 2, A and B). The acceleration of activation kinetics by $\alpha_2\delta$ was also diminished by $\gamma_2$ co-expression (Fig. 4, C and D) and Table I). Finally, data in Fig. 5 strongly suggest that the $\gamma_2$ subunit antagonizes the modulatory effect of $\alpha_2\delta$ on current amplitude of neuronal Ca$^{2+}$ channels. Although the mechanism underlying the $\alpha_2\delta$-dependent inhibitory effect of the $\gamma_2$ subunit is not clear, one of the studies in the $\gamma_1$ null mice suggested that the observed Ca$^{2+}$ channel current increase was due to the increase in channel open probability rather than an increase in the total number of channel and/or single channel conductance (33). Since the $\alpha_2\delta$ subunit is able to increase the open probability of Ca$^{2+}$ channels (35), it is likely that the $\gamma_2$ subunit counteracts the effects of $\alpha_2\delta$ on the open probability of Ca$^{2+}$ channels and thus the Ca$^{2+}$ channel current amplitude. The antagonism between $\alpha_2\delta$ and $\gamma_2$ could be involved in the fine-tuning of Ca$^{2+}$ channel functional activity. In addition, the $\alpha_2\delta$ dependence of the $\gamma_2$ inhibitory effect could eliminate the possibility of nonspecific inhibition of $\gamma_2$ on the expression of neuronal Ca$^{2+}$ channels in our experiments. The recombinant Ca$^{2+}$ channels without $\alpha_2\delta$ did not show significant decrease of current amplitude by co-expression of the $\gamma_2$ subunit (Fig. 5).

Previously, Letts et al. (3) and Klugbauer et al. (10) reported that $\gamma_2$ co-expression shifted the voltage dependence of steady-state inactivation to more negative potentials but had no effect on the current amplitude of $\alpha_{1B}\beta_2\delta$ Ca$^{2+}$ channels expressed in baboon hamster kidney and HEK293 cells, respectively. It is not clear why there is this difference of the $\gamma_2$ effects on Ca$^{2+}$ channels between the previous studies and this report. However, these inconsistencies could be associated to differences in the combination of subunits employed, the expression level of endogenous and exogenous Ca$^{2+}$ channel subunits, the experimental setup for electrophysiological recording, and/or particular physiological conditions such as the phosphorylation status of the channels.

The Modulation of Ca$^{2+}$ Channels by the $\gamma_1$ Subunit—Interestingly, the $\gamma_1$ subunit exerted a modulatory effect similar to that of $\gamma_2$ on neuronal Ca$^{2+}$ channels despite its muscle-specific expression and significant primary sequence divergence between the two isoforms. The $\gamma_1$ subunit decreased current amplitude of both neuronal $\alpha_{1B}$-class (Fig. 2, A and B) and $\alpha_{1A}$-class channels (data not shown), and modulated the activation kinetics of $\alpha_{1B}$-class channels (Fig. 4, C and D) and Table I). Similarly, co-expression of $\gamma_1$ significantly decreased the current amplitude of $\alpha_{1C}\beta_2\delta$ channels in Xenopus oocytes (5). Likewise, as mentioned above, recent electrophysiological studies showed that the current amplitude of skeletal muscle Ca$^{2+}$ channel ($\alpha_{1S}$-class) is significantly increased in the $\gamma_1$ null mice (33, 34). Taken together, these results suggest that the $\gamma_1$ subunit could exert inhibitory effect not only on $\alpha_{1S}$- but also on other Ca$^{2+}$ channels including those of the $\alpha_{1A}$, $\alpha_{1B}$-, and $\alpha_{1C}$-class. However, there have been controversial reports regarding the $\gamma_1$ function. Depending on the subunit composition, $\gamma_1$ co-expression increased or decreased the current amplitude of $\alpha_{1C}$- or $\alpha_{1A}$-class channels expressed in oocytes or HEK 293 cells (4–6, 9). The changes in steady-state activation by $\gamma_1$ co-expression also varied in $\alpha_{1C}$-class channels (5, 6, 9). The $\gamma_1$ co-expression caused a negative shift of steady-state inactivation curves of $\alpha_{1C}$-class channels in some studies (5, 7, 9).

In summary, our study demonstrates the association of the $\gamma_2$ subunit with the neuronal Ca$^{2+}$ channel complex in vivo and indicates an inhibitory function for the $\gamma_2$ subunit.

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