Coexpression of a $Ca_v1.2$ protein lacking an N-terminus and the first domain specifically suppresses L-type calcium channel activity

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Abstract **L-type Ca^2 channels play a critical role in many types of cells, including nerve, muscle and endocrine cells. The most popular and effective tools for analyzing the roles of L-type calcium channels (L-channels) are specific antagonists such as dihydropyrigines. With these drugs however, it is difficult to target specific cells. One solution is to develop a genetically targetable inhibitor coded by DNA. As a candidate for such an inhibitor, a dominant negative mutant of $Ca_v1.2$ was designed by mimicking an ascidian 3-domain-type α1 subunit (that inhibits the full-length subunit's current). The 3-domain-type $Ca_v1.2$ subunit significantly inhibited wild-type $Ca_v1.2$ current, but not other ionic currents such as $Ca_v2.1$ and Na_v channels in *Xenopus* oocyte expression systems. Western blot analysis showed that the expression of the wild-type protein into the plasma membrane was significantly suppressed on coexpression with the truncated protein. These findings support that an N-terminus-truncated mutant could serve as a specific genetically encoded inhibitor for L-channels.**

Key words: Calcium channel; L-type; Truncation; Specific inhibition; *Xenopus* oocyte

1. Introduction

L-type voltage dependent calcium channels are distributed broadly [1,2], and have many roles in cell functions [3], including gene expression [4–6], learning and memory [7]. There are specific drugs for L-type calcium channels (L-channels) such as dihydropyrigine, phenylalkylamines [8] and FPL64176 [9]. These reagents serve as important tools to analyze the roles of L-channels. However, L-channels exist in many types of cells. It is therefore difficult to define their roles in vivo using these drugs. Knock out mice of L-channel α1 subunit genes have already been reported [10]. However, the knocking out of $Ca_v1.2$ in mice is fatal. A new method needs to be established to overcome such problems.

We designed here a dominant negative mutant of the L-channel based on a recent finding in the ascidian Ca^{2+} channel; a 3-domain-type variant of the calcium channel α1 subunit TuCa1 had a significant inhibitory effect on the expression of the full-length calcium channel current both in a *Xenopus* oocyte expression system and ascidian native cells [11]. Since TuCa1 is closely related to L-channels, we designed a similar deletion in the $Ca_v1.2$ mutant by referring to this truncated isoform. We found that the 3-domain-type $Ca_v1.2$ ($\Delta Ca_v1.2$) mutant has a strong inhibitory effect on the wild-type $Ca_v1.2$ channel current. This inhibition was specific to $Ca_v1.2$, since it did not affect the current activity of Na_v channels and $Ca_v2.1$. We thus propose that the dominant negative $Ca_v1.2$ mutant will serve as a useful molecular tool for future studies on L-type Ca^{2+} channels.

2. Materials and methods

2.1. DNA construction

For optimizing translation, KOZAK core sequence [12] was inserted just before the first Met of the α_1 subunits.

A full-length $Ca_v1.2$ cDNA [13] was subcloned into a *Xenopus* expression vector, pSD64TR, at *Kpn*I and *Not*I sites. $\Delta Ca_v1.2$ was designed as in Fig. 1. The 5′-side was amplified using Pfu polymerase (Stratagene) and was joined to the 3′-part of the $Ca_v1.2$ subunit at the *Eco*RI site. A stop codon was introduced into the 3-domain-type mutant (stop$\Delta Ca_v1.2$), as shown in Fig. 4a. A DNA fragment around first Met and stop codon was obtained using Pfu polymerase and joined to the 3′ DNA fragment of $Ca_v1.2$.

The 5′-regions containing putative initial methionines of both the full-length and $\Delta Ca_v2.1$ cDNA were amplified by Pfu polymerase, were connected to the 3′ part of the $Ca_v2.1$ subunit at the *Hin*dIII site, and were subcloned into pSD64TR at *Kpn*I and *Eco*RV sites.

2.2. RNA injection for Xenopus oocytes

Plasmids containing channel subunit cDNA were linearized with *Sal*I (most of cDNAs) or *Not*I (β2b and $Na_v1.4$) and transcribed with SP6 (most of cDNAs) or T7 (β2b) RNA polymerase for injection into *Xenopus* oocytes.

Xenopus oocytes were prepared as described [14]. Synthetic RNA of α1 subunits was injected into an oocyte at a concentration of 0.25 ng/nl. β2b and α2δ subunits of the rabbit Ca^{2+} channel were simultaneously injected at 0.25 ng/nl. In the experiments of coexpression with $Na_v1.4$ RNA, concentrations were 0.15 ng/nl μ1, 0.2 ng/nl α1, β2b and α2δ. RNA concentrations were modified as shown in the figure legends. The volume of RNA solution injected into each oocyte was 50 nl. Injected oocytes were cultured at 18°C. The amount of cRNA

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was roughly adjusted based on the concentration estimated by ethidium bromide staining following RNA electrophoresis. All cRNA injections contained β2b and α2δ. Oocytes from the same batch were recorded within about 5 h between 2 and 4 days after injection.

2.3. Electrophysiology of Xenopus oocytes

Electrical recordings of *Xenopus* oocytes were made as described in [11].

The solutions used for recording Ba^{2+} currents consisted of 20 mM $Ba(OH)_2$, 35 mM NaOH, 77 mM methansulfonate, 2 mM KOH, and 5 mM HEPES (pH 7.4); or 5 mM $Ba(OH)_2$ and 65 mM NaOH. The solution for recording Na^+ currents consisted of 96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl and 5 mM HEPES (pH 7.4). Linear leaks were determined by measuring holding currents. Recordings with large leakages (>200 nA at -70 mV) were discarded. Results are presented as the mean ± S.E.M. (error bars in the figures).

2.4. Transient expression of $Ca_v1.2$ subunits in BHK6 cells

Full-length and $\Delta Ca_v1.2$ were transiently expressed in BHK6 cells [15]. Cell cultures and DNA transfections were carried out as described in [16].

2.5. Whole-cell patch clamp recording

The whole-cell L-type channel currents were recorded with 2 mM Ca^{2+} or 10 mM Ba^{2+} as a charge carrier in a bath solution containing 137 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 2 mM $CaCl_2$ (pH 7.4) at room temperature. The resistance of the microelectrodes ranged from 1.5 to 4 MΩ when filled with 110 mM cesium methanesulfonate, 20 mM TEA, 14 mM EGTA, 5 mM MgATP, 5 mM disodium creatine phosphate, 0.2 mM GTP, and 10 mM HEPES (pH 7.3). Currents were measured as described in [16]. Results are presented as the mean ± S.E.M. Statistical significance was assessed with the Student–Welch unpaired *t*-test. Differences at $P<0.05$ were considered to be significant.

2.6. Western blotting

At 36 h after transfection, cells cultured in 35 mm dishes were incubated on ice for 5 min by replacing with 180 μl of Triton X-100 buffer containing 10 mM Tris–Cl (pH 7.4), 5 mM EGTA, 5 mM EDTA, 120 mM NaCl, 1% (or 0.1%) Triton X-100, 200 nM PMSF, 2 μM leupeptin, and 3 μM pepstatin A. Cells were then mixed and incubated on ice for 5 min. Cell lysates were separated by spinning. Supernatant (150 μl) was added to 4×SDS loading buffer (50 μl). The pellet was added to 1% Triton X-100 buffer (180 μl) and 4×SDS loading buffer (60 μl) and incubated at 37°C for 20 min, then loaded for SDS–PAGE. The rabbit polyclonal antibody CT4 was made to target the C-terminal (aa 2027–2143) of the $Ca_v1.2$ subunit.

3. Results

3.1. $\Delta Ca_v1.2$ inhibited functional expression of full-length $Ca_v1.2$

To make a $Ca_v1.2$ dominant negative mutant, we referred to the sequence of the ascidian calcium channel (Fig. 1). Our previous study showed that a truncated variant of the ascidian L-type-like Ca^{2+} channel TuCa1 starting from the second

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TuCa1     WLTLAQDLANKILLTLFTIEMLVKMYSLGMQQYFVSLFNRFDCF
ΔNTuCa1                IIS2      MLVKMYSLGMQQYFVSLFNRFDCF
                                 *
ΔCaV1.2   WLTEVQDTANKALLALFTAEMLLKMYSLGLQAYFVSLFNRFDCF
                                 *
ΔCaV2.1   WLSDFLYYAEFIFLGLFMSEMFIKMYGLGTRPYFHSSFNCFDCG
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Fig. 1. Peptide sequences of ascidian (TuCa1) and mammalian ($Ca_v2.1$, $Ca_v1.2$) calcium channel α_1 subunits around the IIS2 region. ΔNTuCa1 starts from the Met indicated by asterisks. Other mutants were designed to start from the same position.

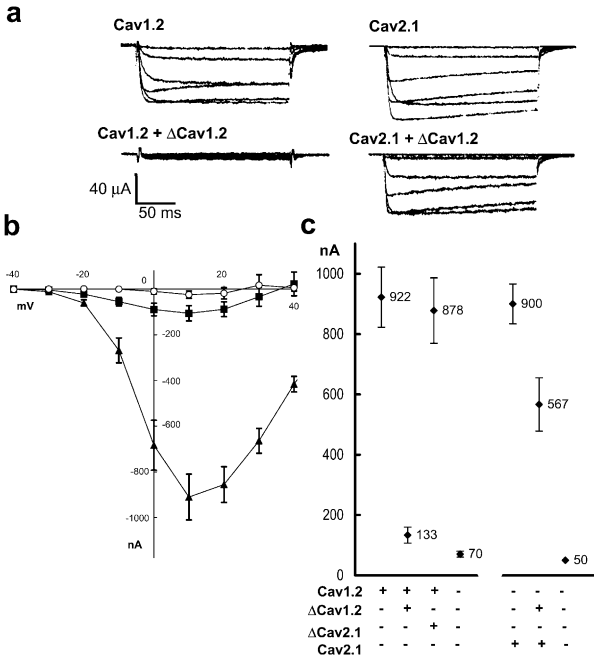

Fig. 2. Biophysical studies of suppressive effect by $\Delta Ca_v1.2$ in *Xenopus* oocytes. $\Delta Ca_v1.2$ significantly reduces expression of $Ca_v1.2$. a: Representative traces of Ba^{2+} currents from oocytes injected with each combination of cRNA as indicated. b: I–V relations of Ba^{2+} currents from cells only expressing $Ca_v1.2$ (triangle; $n=7$), those co-expressing $Ca_v1.2$ and $\Delta Ca_v1.2$ (rectangle; $n=7$) and those only expressing auxiliary subunits (circle; $n=6$). c: Comparison of maximum amplitudes of Ba^{2+} currents among oocytes expressing various combinations of subunits.

domain IIS2 works as the suppressor of functional expression of the full-length protein. We suspected that a similar deleted form of mammalian Ca_v channel may exhibit such inhibition. We thus made a 3-domain-type α_1 mutant starting from the corresponding Met (Fig. 1, asterisked Met).

To test the inhibitory effect of the $\Delta Ca_v1.2$ and $\Delta Ca_v2.1$, full-length α_1 cRNAs were coexpressed with or without 3-domain-type mutants. $\Delta Ca_v1.2$ inhibited almost completely the wild-type $Ca_v1.2$, while $\Delta Ca_v2.1$ had no effect on $Ca_v1.2$ (Fig. 2). On the other hand, $\Delta Ca_v1.2$ had no strong inhibitory activity against the P/Q-type calcium channel subunit $Ca_v2.1$ (Fig. 2a,c). The experiments were repeated four times using other batches of oocytes and similar results were obtained.

3.2. General translation was normal during the inhibition of Ca_v currents by truncated mutants

To test the possibility that the combination of $Ca_v1.2$ and $\Delta Ca_v1.2$ has a strong inhibitory effect on general protein synthesis in *Xenopus* oocytes, we examined the Na^+ channel current derived from the rat $Na_v1.4$ gene when either coexpressed or not with $Ca_v1.2$ channel subunits. At 20 ms depolarization, Na^+ current traces were similar among cells injected with Na^+ channel $Na_v1.4$ only, with $Na_v1.4$ plus $Ca_v1.2$, and with $Na_v1.4$, $Ca_v1.2$ plus $\Delta Ca_v1.2$ (Fig. 3), suggesting that $\Delta Ca_v1.2$ does not suppress general translation in oocytes. The experiments were repeated twice using other batches with similar results.

3.3. Inhibition by $\Delta Ca_v1.2$ occurred after translation

It is possible that $\Delta Ca_v1.2$ inhibits $Ca_v1.2$ expression

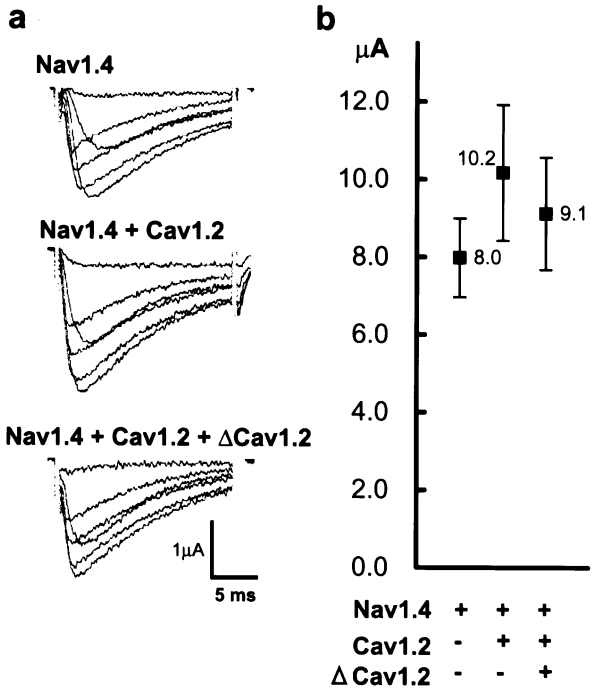

Fig. 3. Absence of inhibition of sodium channel expression. a: Representative traces of $Na_v1.4$ Na^+ currents from *Xenopus* oocytes. b: Comparison of maximum amplitudes of Na^+ currents.

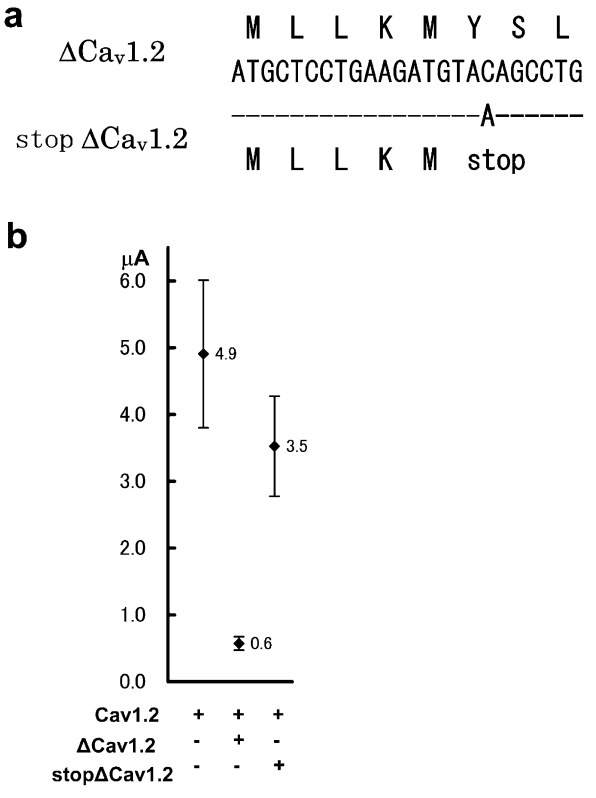

Fig. 4. cRNA having a stop codon just after the first Met did not inhibit $Ca_v1.2$ channel current. a: N-terminal DNA sequence of $\Delta Ca_v1.2$. A point mutation (lower 'A') was introduced. b: Maximum amplitudes of Ba^{2+} currents among oocytes. Concentrations of injected cRNAs were, 0.2 ng/nl for $Ca_v1.2$, and 0.3 ng/nl for stop$\Delta Ca_v1.2$ and $\Delta Ca_v1.2$.

through RNA–RNA interaction. Alternatively, it may have an inhibitory effect at the protein level. To distinguish between these two possibilities, we introduced a stop codon into $\Delta Ca_v1.2$ just after the initial Met (Fig. 4a), retaining all the downstream sequence of $\Delta Ca_v1.2$ (stop$\Delta Ca_v1.2$). Stop$\Delta Ca_v1.2$ did not exhibit an inhibitory effect like $\Delta Ca_v1.2$ (Fig. 4b). This indicates that inhibition by $\Delta Ca_v1.2$ should occur at a step after the translation, not at the RNA level.

3.4. $\Delta Ca_v1.2$ prevented the full-length protein from appearing in membrane fractions

To gain insight into the molecular mechanism of inhibition by $\Delta Ca_v1.2$, we used a transient DNA transfection system. We utilized a mammalian cell line (BHK-6 cells) instead of *Xenopus* oocytes for this experiment, because we could not reliably detect $Ca_v1.2$ protein from *Xenopus* oocyte by Western blot analysis. First, we checked whether inhibition of the full-length channel activity by $\Delta Ca_v1.2$ also occurs in BHK-6 cells, which stably express rabbit β1a and α2δ subunits. Patch clamp recordings showed that coexpression with $\Delta Ca_v1.2$ markedly reduced the amount of current derived from the full-length $Ca_v1.2$ (Fig. 5a,b). By Western blotting of the Triton X-100 soluble fraction of cells of the same batches used for electrophysiology, $Ca_v1.2$ immunoreactivity was shown to be greatly reduced on coexpression with $\Delta Ca_v1.2$. This inhibition seemed dose-dependent: three times more $\Delta Ca_v1.2$ significantly reduced the immunoreactivity of the full-length $Ca_v1.2$. Unexpectedly, the immunoreactivity of $\Delta Ca_v1.2$ was much weaker than the band of full-length $Ca_v1.2$ even though $\Delta Ca_v1.2$ was transfected with a three-fold excess of DNA (Fig. 5c). In addition, the immunoreactivity of $\Delta Ca_v1.2$ in

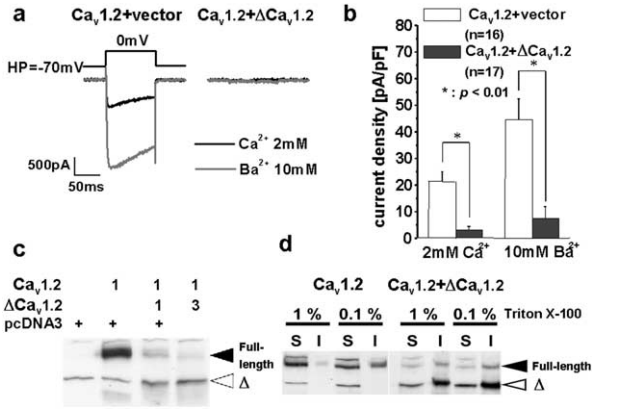

Fig. 5. The suppression of Ca^{2+} channel activity and protein expression by $\Delta Ca_v1.2$ in BHK6 cells. a: Coexpression of $\Delta Ca_v1.2$ caused significant suppression of L-channel current. Representative traces recorded from cells transfected with DNAs as indicated. Ca^{2+} channel current traces were recorded with 2 mM Ca^{2+}(black) or 10 mM Ba^{2+}(gray). b: Current densities of Ca^{2+} or Ba^{2+} current. Asterisks indicate $P < 0.01$. c: $\Delta Ca_v1.2$ suppressed the expression of $Ca_v1.2$. BHK6 cells were transfected with $Ca_v1.2$ plus pcDNA3 or $\Delta Ca_v1.2$ at a molar ratio of 1:1 or 1:3. Soluble fractions of cell lysates were analyzed with anti-CT4 antibody. d: Low solubility of $\Delta Ca_v1.2$. BHK6 cells indicated by Western blot, transfected as indicated at a molar ratio of 1:3, were harvested. Triton X-100 soluble (S) and insoluble (I) fractions were analyzed with anti-$Ca_v1.2$ CT4 antibody. A non-specific band slightly overlapped the signal for $\Delta Ca_v1.2$ in the soluble fraction (c,d). Note that the increased intensity of the band on transfection of $\Delta Ca_v1.2$ which indicates the presence of $\Delta Ca_v1.2$.

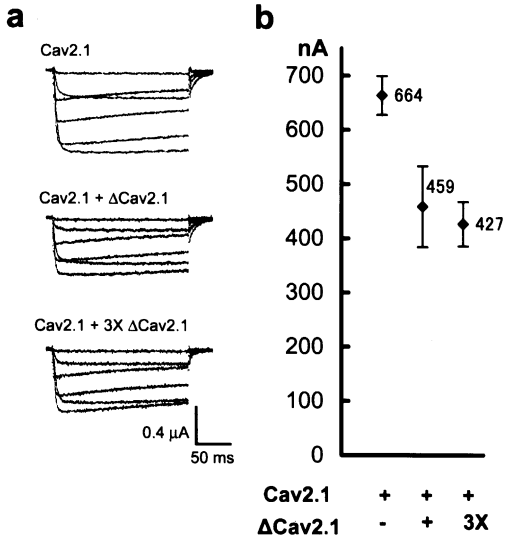

Fig. 6. $\Delta Ca_v2.1$ mutant did not work as a dominant negative mutant. a: Representative traces of Ba^{2+} currents from *Xenopus* oocytes. b: Comparison of maximum amplitudes of Ba^{+} currents. The cRNA concentration is modified as 0.15 ng/nl for $Ca_v2.1$, 0.15 ng/nl or 0.45 ng/nl for $\Delta Ca_v2.1$, 0.15 ng/nl for β2b and 0.15 ng/nl for α2δ.

cells transfected with $\Delta Ca_v1.2$ alone was less than that of $Ca_v1.2$ (data not shown). To check the possibility that $\Delta Ca_v1.2$ is expressed in the insoluble fraction, we harvested the cells on altering Triton X-100 concentrations, and analyzed $Ca_v1.2$ channel immunoreactivity in the insoluble fraction. This analysis indicated that the $\Delta Ca_v1.2$ subunit tended to exist in the insoluble fraction. The immunoreactivity of the full-length $Ca_v1.2$ in the soluble fraction decreased when $\Delta Ca_v1.2$ was coexpressed as already shown (Fig. 5c). In contrast, $Ca_v1.2$ in the insoluble fraction with 1% Triton X-100 increased when $\Delta Ca_v1.2$ was coexpressed (Fig. 5d). In the soluble fractions, there was a non-specific band slightly overlapping with the signal for $\Delta Ca_v1.2$ (Fig. 5c,d). However, the existence of $\Delta Ca_v1.2$ was evident from the increased intensity of the band on transfection of $\Delta Ca_v1.2$, as compared with transfection of the vehicle vector alone.

3.5. $\Delta Ca_v2.1$ did not act as a dominant negative mutant

To know whether a truncated protein lacking an N-terminus and the first domain of other classes of Ca^{2+} channels also show similar negative effect, we made a similar 3-domain mutant for $Ca_v2.1$ (Fig. 1). However, this mutant caused little inhibition of the full-length $Ca_v2.1$ current (Fig. 6). There was still no strong inhibition even when three-times excess amount of $\Delta Ca_v2.1$ cRNA was injected. Similar results were obtained for $Ca_v3.1$ in HEK cells (data not shown). Therefore, suppression by $\Delta Ca_v1.2$ may be mediated by mechanisms unique to $Ca_v1.2$.

4. Discussion

In this study, we designed an inhibitory subunit of $Ca_v1.2$ by mimicking an endogenous calcium channel subunit of ascidian embryos which strongly suppresses the functional expression of the full-length protein [11]. This subunit ($\Delta Ca_v1.2$) significantly inhibited $Ca_v1.2$ channel current in a subtype-specific manner (Fig. 2). Moreover, the inhibition by $\Delta Ca_v1.2$ had no effect on the expression of the Na^{+} channel (Fig. 3), indicating that it is not mediated by inhibition of the general translational machinery. Since the insertion of a premature stop codon into $\Delta Ca_v1.2$ led to a loss of the effect, the inhibition must occur at the protein level (Fig. 4). Western blot analysis indicated that the coexpression of $\Delta Ca_v1.2$ reduced the expression of full-length $Ca_v1.2$ in the Triton X-100 soluble fraction (including the plasma membrane) (Fig. 5). A similar deletion construct for another class of calcium channel, $Ca_v2.1$ however, had no strong inhibitory effect on the full-length $Ca_v2.1$ current (Fig. 6), suggesting that inhibition of the full-length-derived current by the 3-domain-type α1 subunit occurs via a mechanism unique to Ca_v1 class channels.

Familial episodic ataxia type 2 is an autosomal dominant paroxysmal cerebral disorder. Some types of this disorder are caused by one base mutation of the $Ca_v2.1$ gene [17,18]. This mutation results in the translation of a truncated form of $Ca_v2.1$ consisting of the I, II and a part of the III domain. Recently, truncated subunits consisting of domains I and II or domains III and IV of $Ca_v2.2$ have been reported to work as dominant negative [19]. $Ca_v2.2$ mutants are expressed and effect on protein synthesis or protein folding of wild-type channels. In our case, the 3-domain-mutant of $Ca_v2.1$ and $Ca_v3.1$ did not show a significant inhibitory effect on wild-type channels. Therefore, the mechanism of 3-domain-mutant of $Ca_v1.2$ should be different from the other Ca_v dominant negative effects. β-Subunit is needed for the effective expression of current. It is unlikely that $\Delta Ca_v1.2$ scavenges the β-subunit, because it lacks a β-subunit binding region in the I–II loop [20]. Another line of evidence indicates that this inhibition is subtype-specific: $\Delta Ca_v1.2$ did not inhibit $Ca_v2.1$ current.

Truncated K^{+} channels often show dominant negative effects. This can be accounted for by inhibition of coassembly or insertion into the plasma membrane [21]. A similar interpretation does not apply to the Ca_v channel however, because a single Ca_v subunit forms a single pore. Nevertheless, suppression of the full-length protein by $\Delta Ca_v1.2$ suggests that some cooperative interaction among homogenous channels is required for efficient channel assembly or expression. Immunostaining analysis revealed that L-channels are clustered in cell bodies and proximal dendrites in neurons [22]. Recently, a single channel imaging study of $Ca_v1.2$ suggested that $Ca_v1.2$ molecules form clusters as α1 subunit themselves through self-aggregation [23]. These findings are consistent with the above-mentioned idea.

Our $\Delta Ca_v1.2$ mutant inhibits specifically $Ca_v1.2$ channel current. Although the mechanisms of suppression require further study, this mutant will be potentially useful for analyzing the roles of L-channels. We are currently making transgenic mice which express this $\Delta Ca_v1.2$ mutant only in certain types of neurons to better define the role of the L-channel in neural development and function.

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