DHP-insensitive L-type-like Ca channel of ascidian acquires sensitivity to DHP with single amino acid change in domain III P-region

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Abstract TuCa1, an ascidian homolog of L-type Ca channel α_1-subunit, has many critical sites required for binding 1,4-dihydropyridines (DHPs), but is insensitive to DHPs and methyl 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxylate (FPL-64176). We have substituted Ser for Ala^{1016} at the P-region of domain III in TuCa1 (TuCa1/A1016S) and functionally expressed the channel in *Xenopus* oocyte along with rabbit α_2/δ and β_{2b}. TuCa1/A1016S has gained DHP sensitivity as high as that of a mammalian neuronal L-type Ca channel (rbCII), but remained resistant to FPL-64176. These results reinforce the view that Ser^{1016} in TuCa1/A1016S participates in DHP binding, but there exist other novel sites that fully acquire sensitivity to FPL-64176.

Key words: Ca channel; 1,4-dihydropyridine; FPL-64176; Ascidian, L-type

1. Introduction

Voltage-dependent Ca channel gets opened by membrane depolarization and selectively permeates Ca, thus modulates a variety of cellular functions such as synaptic transmission, secretion, and muscle contraction [1]. Ca channels are complex proteins that consist of a pore-forming α_1 subunit, disulfide-linked transmembrane complex of α_2 and δ subunits (α_2/δ), intracellular β subunit, and γ subunit [1,2]. α_1 subunits are further divided into five major subtypes based on biophysical and pharmacological characteristics: L (α_{1C}($Ca_V1.2$), α_{1D}($Ca_V1.3$), α_{1S}($Ca_V1.1$), α_{1F}($Ca_V1.4$)); N (α_{1B}($Ca_V2.2$)); P/Q (α_{1A}($Ca_V2.1$)); R (α_{1E}($Ca_V2.3$)); and T (α_{1G}($Ca_V3.1$), α_{1H}($Ca_V3.2$), α_{1I}($Ca_V3.3$)) [3,4]. Dihydropyridine (DHP) is a classical Ca^{2+} channel antagonist that binds to L-type Ca^{2+} channel α_1 subunits with high affinity [5,6].

We have cloned TuCa1, a homolog of L-type Ca channel α_1 subunit, from the tadpole of ascidian species, *Halocynthia roretzi* [7]. TuCa1 conserves nearly all of the sites that are crucial for binding DHP [7]. However, the channel is insensitive to DHPs [8]. A number of amino acid sequences of TuCa1 are different in the conserved region of mammalian α_1 subunits, which include DHP-insensitive types. Based on this observation, Ser^{1115} has been substituted with Ala in the pore-forming region (P-region) between IIIS5 and IIIS6 of rbCII ($Ca_V1.2$) [9]. This substitution has remarkably decreased sensitivity of the channel to DHPs and methyl 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxylate (FPL-64176), a benzoylpyrrole-type Ca channel agonist [9].

In the present study, we have reversed substitution of amino acids in TuCa1 from ascidian type to a mammalian type. We show that a single amino acid substitution of TuCa1 recovers full sensitivity to DHP agonist, but not to FPL-64176.

2. Materials and methods

2.1. Site-directed mutagenesis in TuCa1

TuCa1 in pSD64TF, a modified version of pSP64 (Promega, Madison, WI, USA), was kindly provided by Dr. T.P. Snutch [7]. To replace Ala^{1016} with serine (A1016S), a sense primer CTGACGCTCTTTGTTGTT**T**CTACGTTCGAAG (A1016Sforward) was designed with its antisense primer CTTCGAACGTAG**A**AACAACAAAGAGCGTCAG (A1016Sreverse). The bold letter indicates a change from the wild-type TuCa1 sequence. After amplification of *Avr*II (2520)-A1016Sreverse and A1016Sforward-*Acc*I(4117) fragments, the *Avr*II-*Acc*I fragment was amplified from the two fragments above and subcloned into pCR2.1 using TA Cloning Kit (Invitrogen, San Diego, CA, USA). The *Mun*I(2928)-*Acc*I fragment of the plasmid was introduced into IIIS5-S6 linker of TuCa1-pBluescript and the full-length TuCa1/A1016S was inserted into pSD64TF via *Not*I and *Kpn*I.

2.2. RNA injection into Xenopus oocytes

The above plasmids were linearized with *Sal*I, transcribed by SP6 RNA polymerase, and injected into *Xenopus* oocyte. The cDNA clones that were co-expressed with either the wild-type or mutant TuCa1 were comprised of pBH17, a rabbit β_{2b} subunit (#X64298) kindly provided by Dr. V. Flockerzi [10], and pSPCA1, a rabbit α_2/δ subunit (#M21948) kindly provided by Dr. T. Tanabe [11,12]. Rat brain α_{1C} subunit (rbCII, $Ca_V1.2c$, #M67515), kindly provided by Dr. T.P. Snutch [13], in pSD64TR [14] was co-expressed with β_{2b} and α_2/δ as a control.

Xenopus oocytes were prepared as described previously [7]. The concentration of the injected synthetic RNA of α_1 subunits was 0.5 ng/nl. The concentration of the injected β_{2b} and α_2/δ subunits of rabbit VDCC was 0.25–0.5 ng/nl. The injected volume of RNA solu-

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Abbreviations: DHP, dihydropyridine; FPL-64176, methyl 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxylate; Bay k 8644, 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester; DMSO, dimethyl sulfoxide

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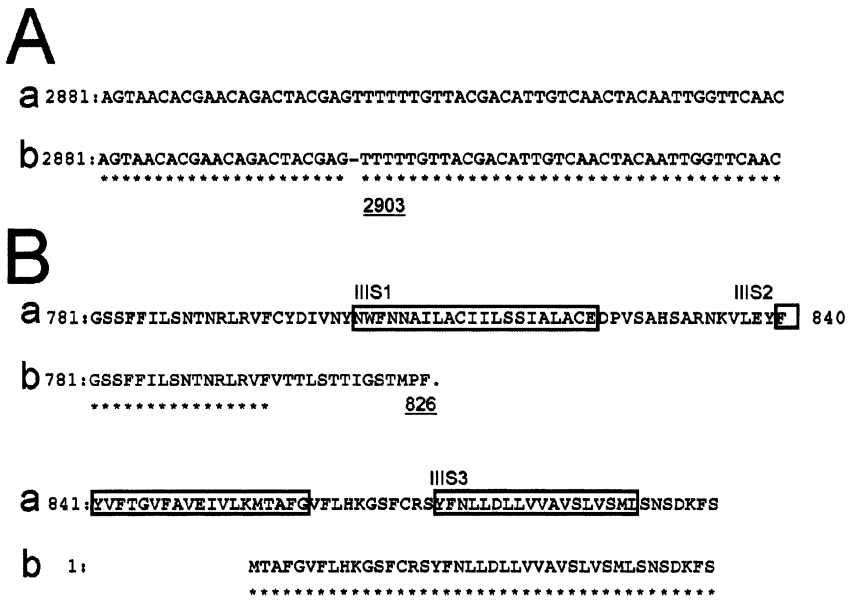

Fig. 1. A: Partial nucleotide sequence of TuCa1 or TuCa1/A1016S which has a deletion of T^{2903} (b) in this study is aligned with that of normal TuCa1 (a) (accession number AB013604). B: Frame shift and truncation caused by the deletion of T^{2903} and a proposed restart site of the C-terminal half of TuCa1 are illustrated (b). Normal TuCa1 (a) is aligned with the frame-shift mutant. Transmembrane segments are boxed. Identical nucleotide and amino acid between the mutant and normal TuCa1 are indicated with an asterisk.

tion was 50–100 nl per oocyte. The oocytes were incubated for 2–5 days at 18°C prior to recording.

2.3. *Electrophysiology of* Xenopus *oocytes*

The electrical activity of oocytes was recorded as described previously [7]. To record Ba^{2+} currents, bath solution was composed of the following (in mM): $Ba(OH)_2$, 20; NaOH, 35; KOH, 2; methane sulfonate, 77; HEPES, 5; niflumic acid, 0.5 (pH 7.4). To eliminate Cl current that gets activated by divalent cations, 50 nl of 50 mM Na_4BAPTA with 10 mM HEPES (pH 7.0) was microinjected into each oocyte 5–30 min before the recording [15]. The currents were recorded and analyzed with Pulse+PulseFit 8.09 (Heka Elektronik, Lambrecht/Pfalz, Germany) and IGOR Pro Ver 3.0 (WaveMetrics, OR, USA), respectively. The results are presented as means ± S.E.M.

2.4. *Materials*

S-(-)-Bay k 8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester) and FPL-64176 (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in EtOH and stored at −20°C as 3 mM stock solutions. Each drug was previously diluted into a 10th of the final concentration and was directly added to the bath solution.

3. Results

3.1. *Electrophysiological properties of TuCa1 and TuCa1/A1016S*

We replaced Ala^{1016} with Ser in IIIS5-S6 linker of TuCa1, which is the opposite of the previous experiment, in which Ser^{1115} in rbCII was replaced with Ala [9]. Ser is conserved in α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E} and α_{1S}, but not in TuCa1 or CyCaα_1, a jellyfish α_1 Ca channel. We unexpectedly found that both the wild-type and mutant TuCa1 have a deletion of T at 2903, which might cause a frame shift just before domain III and a truncation at Arg^{826} (Fig. 1). Despite the mutation, these clones were functionally expressed in *Xenopus* oocyte. This point will be discussed later.

Either the wild-type or mutant TuCa1 generated currents when co-expressed with accessory subunits, namely, rabbit β_{2b} and rabbit α_2/δ in *Xenopus* oocyte. We used Ba^{2+} ions as

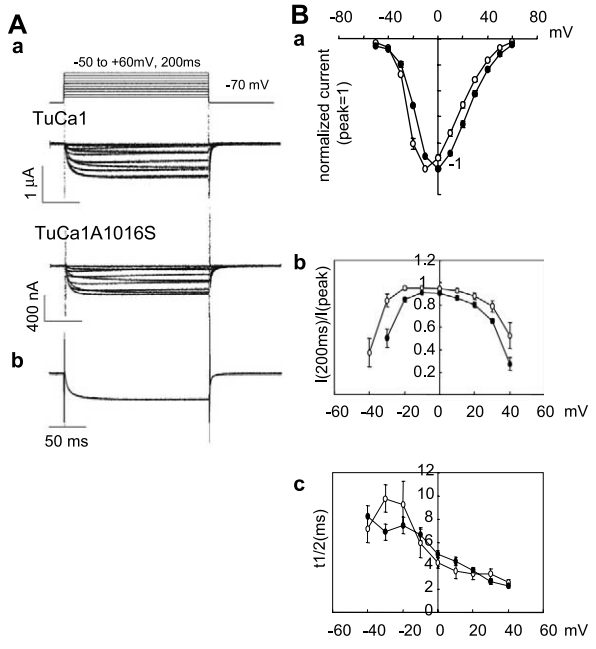

Fig. 2. Electrophysiological properties of Ba^{2+} currents derived from wild-type TuCa1 or TuCa1/A1016S co-expressed with rabbit β_{2b} and α_2/δ in *Xenopus* oocyte. A: (a) Representative Ba^{2+} current of TuCa1 and TuCa1/A1016S. Protocol for voltage clamp is shown at the top. (b) Ba^{2+} currents at −10 mV of TuCa1 (gray) and TuCa1/A1016S (black). The traces were normalized and superimposed. B: Current–voltage relationship (*I–V*) (a), ratios of current amplitude at the end of step pulse (200 ms) to peak amplitude (b) and time to reach half of the peak current amplitude during the step pulses of 200-ms interval (c) plotted against membrane potential. Open and closed circles indicate wild-type and mutant TuCa1, respectively. In (a)–(c), $n = 4$ for TuCa1, $n = 5$ for TuCa1/A1016S.

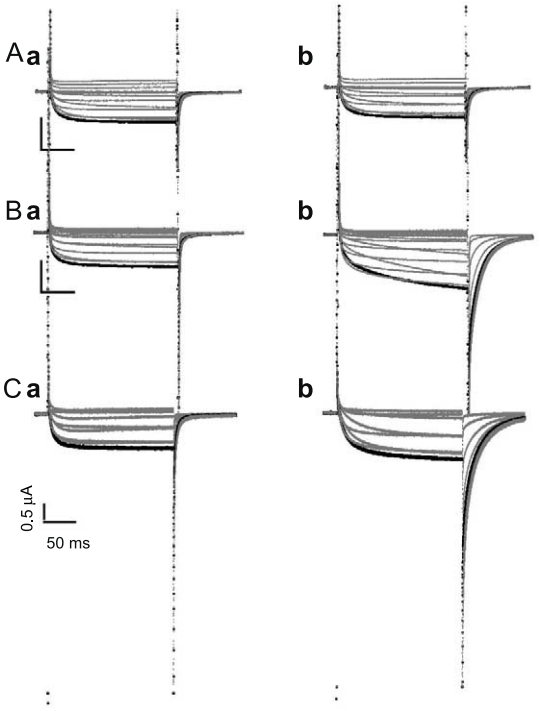

Fig. 3. Representative traces of Ba^{2+} currents derived from TuCa1 (A), TuCa1/A1016S (B) and rbCII (C) in the absence (a) or presence (b) of 8 μM S-(-)-Bay k 8644 activated by the protocol shown in Fig. 2A(a). The peak current in each panel is traced in black.

current carriers in order to reduce Ca^{2+}-dependent inactivation. A comparison of kinetics between both channels is shown in Fig. 2. The current–voltage relationship (*I–V*) revealed that the peak current amplitude of TuCa1/A1016S was found around 0 mV, which was shifted to the right of that of wild-type TuCa1 (Fig. 2B(a)). However, both channels showed almost the same kinetics (Fig. 2B(b,c)). Furthermore, superimposed traces of the normalized current show an identical waveform when voltage-clamped at −10 mV (Fig. 2A).

3.2. TuCa1/A1016S gained DHP sensitivity

The Ba^{2+} current amplitude increased (1.43 ± 0.14)-fold in rbCII with the application of 8 μM S-(-)-Bay k 8644, a DHP agonist (Figs. 3C and 4A), but decreased with either 8 μM nitrendipine or nifedipine, the DHP antagonists (data not shown). Because 8 μM agonist optimally enhanced the rbCII-derived current in *Xenopus* oocyte (data not shown), all experiments were performed under the same condition. Wild-type TuCa1 was unresponsive to S-(-)-Bay k 8644 (Fig. 3A) and a slight decline of the current was observed (Fig. 4A, (0.89 ± 0.06)-fold). Wild-type TuCa1 was also resistant to 4 μM nifedipine and 8 μM nitrendipine (data not shown). In contrast, TuCa1/A1016S significantly increased sensitivity to DHP, similar in fashion to rbCII; the current amplitude increased by (1.49 ± 0.21)-fold with the application of agonists (Fig. 3 and 4B) and decreased with antagonists (data not shown). The magnitude of changes in current amplitudes was as great as that in rbCII. In addition, TuCa1/A1016S showed leftward shifts in the *I–V* and peak current amplitude (Fig. 4C). Deceleration of deactivation kinetics is another hallmark of DHP action on L-type calcium channel. Thus, the deactivation kinetics was analyzed by measuring the decay time constant of tail currents. Both TuCa1 and TuCa1/A1016S currents were fitted by a single exponential, whereas rbCII was fitted by a double exponential (Fig. 4B(b)). Upon application of S-(-)-Bay k 8644, TuCa1/A1016S showed about five-fold increase in the time constant (Fig. 4B(a)).

3.3. Current amplitude of TuCa1/A1016S was not enhanced by FPL-64176

FPL-64176, a benzoylpyrrole-type Ca channel agonist, binds to the Ca channel at a different binding site than DHPs [16]. It has been reported that rbCIIS1115A only weakly enhances the current with an application of FPL-64176 and DHP agonist compared to the wild-type rbCII channel [9]. In our study, FPL-64176 increased the current of wild-type rbCII by (1.84 ± 0.77)-fold (Figs. 5C and 6), which is a greater increase than S-(-)-Bay k 8644. However, application of 10 μM FPL-64176 did not enhance the current of the wild-type TuCa1 ((0.95 ± 0.02)-fold, Figs. 5A and 6)) or TuCa1/A1016S ((1.04 ± 0.1)-fold, Figs. 5B and 6)). TuCa1/A1016S showed a mild deceleration of the tail current with an application of FPL-64176 (Fig. 6A).

4. Discussion

The wild-type and mutant TuCa1 clones unexpectedly

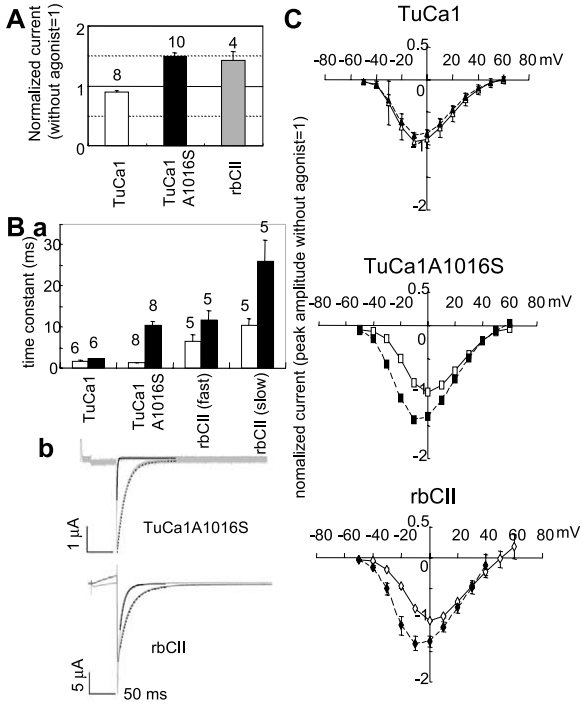

Fig. 4. Effects of 8 μM S-(-)-Bay k 8644 on peak current amplitudes (A), time constant of deactivation (B), and *I–V* (C) of Ba^{2+} current derived from TuCa1, TuCa1/A1016S and rbCII. A: Each bar indicates the peak current amplitude with presence of agonist, normalized by amplitude without. B: (a) Tail currents at −70 mV after the step pulse (50 ms, 50 mV) were analyzed by single (TuCa1 and TuCa1/A1016S) or double (rbCII) exponential fit. Time constants are plotted against membrane potential before (white) and after (black) the application of the agonist. Number of cells is indicated above the bars. (b) Representative examples of exponential fit of the tail current of TuCa1/A1016S and rbCII either before (bold curve) or after (dotted curve) agonist application. C: *I–V* relations of Ba^{2+} current before (white) and after (black) the application of agonist.

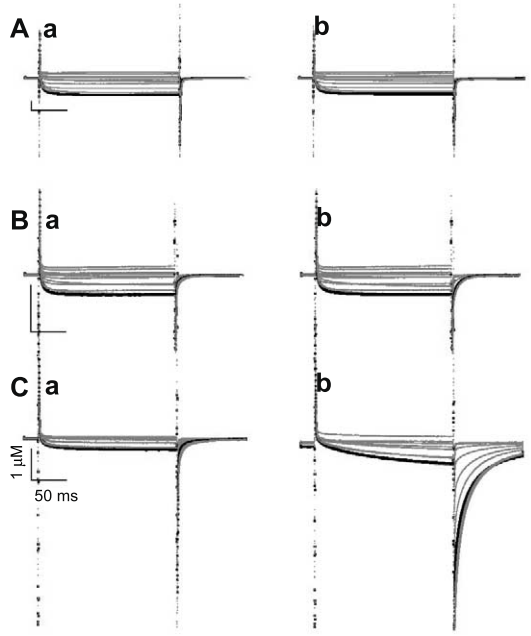

Fig. 5. Representative traces of Ba^{2+} currents derived from TuCa1 (A), TuCa1/A1016S (B) and rbCII (C) in the absence (a) or presence (b) of FPL-64176 at 10 μM. The same protocol as shown in Fig. 2A(a) was used. The peak current in each panel is traced in black.

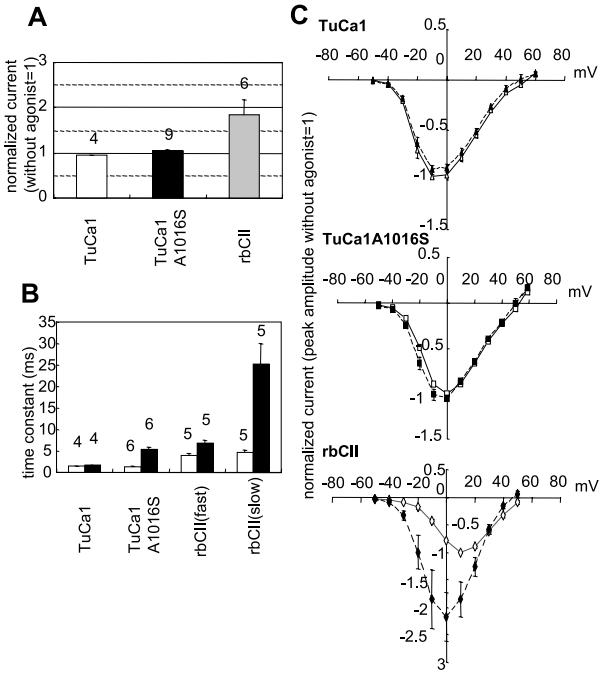

Fig. 6. Effects of 10 μM FPL-64176 on peak amplitudes (A), time constant of deactivation (B) and *I–V* (C) of Ba^{2+} current derived from TuCa1, TuCa1/A1016S and rbCII. A: Each bar indicates the peak current amplitude with presence of FPL-64176, normalized by amplitude without. B: Tail currents were recorded and analyzed as in Fig. 4B. Time constants before (white) and after (black) the application of the agonist. Number of cells is indicated above the bars. C: *I–V* relationships of Ba^{2+} current before (white) and after (black) the application of agonist.

showed a deletion of T at 2903, which may cause a frame shift and truncation at Arg^{826} (Fig. 1A). However, these clones are functionally expressed and acquired DHP sensitivity by the mutation of Ala^{1016} downstream of a site that corresponds to the frame shift. One possibility that accounts for a functional expression is that a complementary polypeptide gets translated from a Met site downstream of the site of frame shift (Fig. 1B), which then co-assembles with the truncated polypeptide and forms a complete channel. It has been reported on cloned $Ca_v1.1$ that two polypeptides get produced from a frame-shifted RNA and successfully reconstitute gating charges in the muscle cell of dysgenic mouse [17]. Another possible mechanism is RNA polymerase slippage, which is reported to occur in *Escherichia coli dna*X mRNA [18]. In fact, the frame shift occurs in the poly Ts (6 Ts) in TuCa1 plasmids, whereas the RNA polymerase slips at a run of 9 Ts in *dna*X. This phenomenon is probably worth another detailed study, yet we choose not to investigate beyond this point in this paper. It is unlikely that this phenomenon affects the major conclusions of this study.

DHP-binding sites have been determined by photo-affinity labeling, radio-ligand binding, chimera, and alanine-scanning mutagenesis based on sequence differences between α_{1C} (or α_{1S}) and DHP-insensitive α_{1A} subunit [19–23]. Alanine-scanning mutagenesis of IIIS6 and IVS6 has shown that some conserved amino acids in α_{1C} and α_{1A} are required for high-affinity binding of Ca channel antagonists [24]. Recently, substitutions of Ala for Phe^{1112}, Ser^{1115} or both in the IIIS5-S6 linker of rbCII have been shown to reduce the sensitivity to DHPs and FPL-64176 [9,25]. Phe, but not Ser, is conserved in TuCa1 and jellyfish CyCaα_1, both of which are DHP-insensitive [9]. In this study, we have shown that reverse mutation from Ala^{1016} to Ser increases DHP sensitivity in TuCa1 as much as that observed in rbCII (Figs. 3 and 4). This result reinforces the view that the Ser in the pore region is a critical determinant for DHP actions.

FPL-64176 and Bay k 8644 are known to bind to different sites on the Ca channel [26,27] and they interact with each other allosterically [16,28,29]. TuCa1/A1016S showed high DHP sensitivity, but did not show increase in the current amplitude by FPL-64176 (Figs. 5 and 6). However, tail current kinetics of TuCa1/A1016S became slightly slower by FPL-64176, suggesting its mild agonist action. It is thus unlikely that TuCa1/A1016S fails to bind FPL-64176. To gain full agonist action of FPL-64176, Ser and Phe [25] in the P-region of domain III may need to interact with other novel amino acid(s) that is (are) not conserved in TuCa1.

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