Effect of Ca\textsubscript{v}β Subunits on Structural Organization of Ca\textsubscript{v}1.2 Calcium Channels

Evgeny Kobrinsky\textsuperscript{*,} Parwiz Abrahimi\textsuperscript{*,} Son Q. Duong, Sam Thomas, Jo Beth Harry, Chirag Patel, Qi Zong Lao, Nikolai M. Soldatov\textsuperscript{*}

National Institute on Aging, National Institutes of Health, Baltimore, Maryland, United States of America

Abstract

Background: Voltage-gated Ca\textsubscript{v}1.2 calcium channels play a crucial role in Ca\textsuperscript{2+} signaling. The pore-forming α\textsubscript{1C} subunit is regulated by accessory Ca\textsubscript{v}β subunits, cytoplasmic proteins of various size encoded by four different genes (Ca\textsubscript{v}β\textsubscript{1} - β\textsubscript{4}) and expressed in a tissue-specific manner.

Methods and Results: Here we investigated the effect of three major Ca\textsubscript{v}β types, β\textsubscript{1B}, β\textsubscript{3D} and β\textsubscript{4}, on the structure of Ca\textsubscript{v}1.2 in the plasma membrane of live cells. Total internal reflection fluorescence microscopy showed that the tendency of Ca\textsubscript{v}1.2 to form clusters depends on the type of the Ca\textsubscript{v}β subunit present. The highest density of Ca\textsubscript{v}1.2 clusters in the plasma membrane and the smallest cluster size were observed with neuronal/cardiacoβ present. Ca\textsubscript{v}1.2 channels containing β\textsubscript{4}, the predominant Ca\textsubscript{v}β subunit of vascular smooth muscle cells, were organized in a significantly smaller number of larger clusters. The inter- and intramolecular distances between α\textsubscript{1C} and Ca\textsubscript{v}β in the plasma membrane of live cells were measured by three-color FRET microscopy. The results confirm that the proximity of Ca\textsubscript{v}1.2 channels in the plasma membrane depends on the Ca\textsubscript{v}β type. The presence of different Ca\textsubscript{v}β subunits does not result in significant differences in the intramolecular distance between the termini of α\textsubscript{1C} but significantly affects the distance between the termini of neighbor α\textsubscript{1C} subunits, which varies from 67 Å with β\textsubscript{1B} to 79 Å with β\textsubscript{3}.

Conclusions: Thus, our results show that the structural organization of Ca\textsubscript{v}1.2 channels in the plasma membrane depends on the type of Ca\textsubscript{v}β subunits present.

Introduction

Voltage-gated Ca\textsubscript{v}1.2 calcium channels react to membrane depolarization by creating a rapid and transient increase in intracellular free Ca\textsuperscript{2+} concentration, thereby playing an essential role in initiation of calcium signaling in a wide variety of cells [1]. In order to exhibit this function, Ca\textsubscript{v}1.2 calcium channels require association of the pore-forming α\textsubscript{1C} subunit with accessory Ca\textsubscript{v}β and Ca\textsubscript{2δ} subunits as well as calmodulin. Calcium channels are clustered rather than evenly distributed along the surface membrane of neurons [2–4] and cardiac myocytes [5–7]. Single-molecule imaging of the functional recombinant EYFP-N1-α\textsubscript{1C}β\textsubscript{2D}/Ca\textsubscript{2δ} channels revealed clusters composed of ~40 channels [8]. In neuronal cell bodies and proximal dendrites in hippocampus and cerebellar cortex, Ca\textsubscript{v}1.2 clusters of 1.5–2 μm in diameter were observed with anti-α\textsubscript{1C} antibody [9]. Using electron microscopy in bird and amphibian cardiac muscle [5,6] and immuno-gold labeling in mammalian ventricular myocytes [7,10] it was shown that Ca\textsubscript{v}1.2 clusters are loosely tethered to ryanodine receptors (RyR) of the sarcoplasmic reticulum. Although association of calcium channels and ryanodine receptors appears to be weaker in cardiac myocytes than in skeletal muscle [11] and may involve different mechanisms of coupling [12], Ca\textsubscript{v}1.2 clustering is essential for excitation-contraction coupling [13,14].

Little is known about the factors affecting the structure of Ca\textsubscript{v}1.2 clusters or the mechanisms of their formation. Because the carboxyl-terminal “IQ” region of α\textsubscript{1C} mediate the calmodulin-dependent Ca\textsuperscript{2+}-induced inactivation of the channel [15–18], it is reasonable to suggest that both calmodulin and the cytoplasmic 750-amino acid C-tail of α\textsubscript{1C} have a role in the formation and maintenance of the Ca\textsubscript{v}1.2 clusters. Indeed, a splice variant of α\textsubscript{1C} (α\textsubscript{1C,361}) deprived of IQ does not show a distinct tendency to form clusters [19]. The role of IQ sequences in intermolecular interactions between neighboring α\textsubscript{1C} molecules was experimentally confirmed in recent diffraction study [20]. The impact of bulky cytoplasmic Ca\textsubscript{v}β subunits on Ca\textsubscript{v}1.2 structure and clustering is not known. Ca\textsubscript{v}β subunits are important differential modulators of the electrophysiological properties of calcium channels [21–23]. These peripheral proteins of variable size are encoded by four different genes (Ca\textsubscript{v}β\textsubscript{1} - β\textsubscript{4}), some of them being subject to alternative splicing [24]. They have a common binding site in the cytoplasmic linker between repeats I and II of α\textsubscript{1C}.
known as the α-interaction domain (AID) [25]. Here, we applied total internal fluorescence reflection (TIRF) and three-color FRET microscopy to assess the effects of Ca,β on cluster size and density of Ca,1.2 as well as to measure inter- and intramolecular distances between the N- and C-termini of α1C and the N-tails of β1b, β2d and β3. Our results demonstrated that Ca,1.2 channels form plasma membrane clusters and revealed the effect of the type of Ca,β present on molecular distances and packing of the channels.

Results

Differential effect of Ca,β subunits on cluster organization of Ca,1.2 channels

Ca,1.2 calcium channels retain functional activity after fusion of fluorescent proteins to the N- and C-termini of α1C and to the N-terminus of Ca,β. In our experiments, we labeled α1C at the N-tail with monomeric mVenus (Y22C) and/or at the C-tail with monomeric mCerulean (C284F) [26]. To investigate the effect of Ca,β subtype on size and density of Ca,1.2 clusters, we chose three major Ca,β variants, neuronal/cardiac β1b [27], cardiac β2d [28,29] and neuronal/cardiac/vascular β3 [30–32], which is the predominant Ca,β subunit in vascular smooth muscle cells [31,33]. The more commonly used β2a was excluded from the experiments because its N-tail is palmytoylated and anchored to the inner leaflet of the plasma membrane.

Fluorescent microscopy is a convenient approach to detect clusters of recombinant calcium channels as fluorescent foci or groupings of labeled molecules [34]. In this study, we used TIRF microscopy to visualize Ca,1.2 clusters on the basol plasma membrane. Wavelet transform was used for the detection of clusters (see Methods and Figure 1A) to estimate the effect of the type of Ca,β present on the Ca,1.2 clusters size (Figure 1B) and density (defined here as number of clusters per μm² of the plasma membrane, Figure 1C). The smallest Ca,1.2 clusters were observed with β1b present. Ca,1.2 clusters were significantly (P<0.001) larger with β2d (by ~20%) and β3 present (by ~30%) (Figure 1B). We also found that the average density of the α1C/β1b clusters in the plasma membrane was 2.5 times higher (P<0.01) than α1C/β2d, with β3d again taking an intermediate value (Figure 1C). Thus, Ca,β subunits differentially regulate the architecture of the Ca,1.2 clusters.

In principle, the close proximity of channels within a cluster may generate intermolecular FRET between the V and C fluorophores of neighboring α1C/C channels. This intermolecular FRET should be absent in clusters, where only intramolecular FRET should occur. The α1C/α1C/β2d/β3 channel was expressed in COS1 cells and two-color TIRF-FRET was measured inside and outside of clusters identified by wavelet transform. Based on FRET efficiency, a V-C distance (r)-frequency histogram of the total number of pixels within clusters revealed a possible bi-modal distribution, where a second (intramolecular) component of FRET is seen within clusters (Figure 2A) but not outside of the clusters (Figure 2B). Because TIRF microscopy captures only a small fraction of the cell plasma membrane, we used epifluorescent three-color FRET microscopy to quantitatively analyze the effect of Ca,β subtype on inter- and intra-molecular distance of Ca,1.2 channels.

The type of Ca,β present does not affect intramolecular distance between the N- and C-termini of the α1C subunit

We investigated the effect of Ca,β subtype on molecular distances in Ca,1.2 channels by three-color FRET between α1C/β1b and tagRFP (Rβ) fused to N-termini of β1b, β2d and β3. The advantage of three-color FRET cell microscopy applied to multisubunit complexes is that the method simultaneously detects the relative arrangement of the three different fluorophores (C, V and R) at a distance ~2×R̅, where R̅ is the Förster radius (R̅(C→V) = 53 Å; R̅(V→R) = 58 Å; R̅(C→R) = 51 Å). Both mCerulean and mVenus are close analogs of GFP and can be approximated by a cylinder of 32×48 Å [35]. However, tagRFP [36], a monomeric analog of eqFP611, is larger in size and can be approximated by a cylinder of 34×54 Å [37]. Use of monomeric forms of fluorescent proteins excludes artifacts due to dimerization after expression [38]. The labeled constructs were co-expressed with Rβ in two different combinations as shown in Figure 3, and three-color FRET was measured using a multicube system [39]. Although membrane potential was not controlled during experiments, it was found to be on average ~10.0±3.3 mV (n = 5) indicating that channels were predominantly in an inactivated state. In each fluorescent cell image, region of interest (ROI) was determined according to [42] (Figure 4, middle panels) and multicolor FRET was measured using a multicube system [39]. Use of monomeric forms of fluorescent proteins excludes artifacts due to dimerization after expression [38]. The labeled constructs were co-expressed with Rβ in two different combinations as shown in Figure 3, and three-color FRET was measured using a multicube system [39]. Although membrane potential was not controlled during experiments, it was found to be on average ~10.0±3.3 mV (n = 5) indicating that channels were predominantly in an inactivated state. In each fluorescent cell image, region of interest (ROI) was determined according to [42] (Figure 4, middle panels) and...
then converted to the distance \( r \) between donor and acceptor (right panel) according to [43].

Results of our measurements revealed that the tested Ca\(_b\) subunits did not affect intramolecular distance between the N- and C-termini of \( \alpha_1C \). Measurement of FRET in the double-labeled V\( \alpha_1CC \) co-expressed with \( \alpha_2d \) and R\( \beta_1b, R\beta_2d \) or R\( \beta_3 \) showed that the intramolecular \( r_{V-C} \) distance \( \approx 6.8 \) nm (light gray bars) was observed both inside and outside clusters, while intramolecular \( r_{V-C} \) distance \( \approx 8.1 \) nm was observed only in clusters (dark gray).

Fragment of \( \beta_2 \) subunits which does not bind to AID, but interacts with the IQ region of the \( \alpha_1C \) subunit C-terminus, facilitates voltage gating and stimulates surface expression of the channel [44]. Results of FRET measurements showed \( r_{C-V} = 68 \pm 1 \) A (n = 22), essentially the same distance as that estimated when AID was occupied by Ca\(_b\). Taken together, these results of our study suggest that type of Ca\(_b\) subunits present does not significantly affect the intramolecular distance between the N- and C-termini of \( \alpha_1C \) in Cav1.2 calcium channels.

Intermolecular distance between the \( \alpha_1C \) subunit N- and C-termini depends on the type of Ca\(_b\) present

Fitting of FRET data obtained with \( \beta_2d \) and \( \beta_3 \) to a sum of two Gaussian distributions (Table 1) revealed a statistically significant second component of V\( \alpha_1CC \) FRET (Figure 5). Arising from neighboring V\( \alpha_1CC \) molecules, this FRET provided estimates for the intermolecular distances \( r_{C-V} \) that were significantly different for \( \beta_2d \) (72 \( \pm \) 3 A, n = 5) and \( \beta_3 \) (77 \( \pm \) 3 A, n = 6). To verify our intermolecular distance measurements, we co-expressed a mixture of V\( \alpha_1C \) and \( \alpha_1CC \) along with R\( \beta_1b, R\beta_2d \) or R\( \beta_3 \). Any FRET between V and C in this recombinant system must be intermolecular FRET between termini of neighboring channels. Results, presented in Table 1, showed that intermolecular distances \( r_{C-V} \) measured in these complexes with \( \beta_2d \) (72 \( \pm \) 2 A, 

Figure 2. Intramolecular vs. intermolecular FRET in V\( \alpha_1CC \) revealed in TIRF images. V\( \alpha_1CC \), \( \alpha_2d \) and \( \beta_3 \) were co-expressed in COS1 cells. Two-color FRET was measured in TIRF images and converted into distances \( r \) between V and C as described in Methods. Shown are normalized cumulative histograms (n = 11) for \( r \) calculated for ROI inside clusters (A, total number of pixels m = 231) and outside clusters (B, m = 3908) identified by wavelet transform. The same intramolecular \( r_{V-C} \) distance \( \approx 6.8 \) nm (light gray bars) was observed both inside and outside clusters, while intramolecular \( r_{V-C} \) distance \( \approx 8.1 \) nm was observed only in clusters (dark gray).

Figure 3. Investigated combinations of the labeled \( \alpha_1C \) and \( \beta \) subunits for three color FRET measurements. V\( \alpha_1CC \) and R\( \beta \) (A) and V\( \alpha_1C, \alpha_1CC \) and R\( \beta \) (B) were co-expressed with \( \alpha_2d \) (not shown). Arrows indicate revealed intramolecular and intermolecular distances.
CA1.2 Structure: CAβ Is Effect

A

\[ V_{\alpha_{1C}C+R\beta_{1b}} \]

Relative frequency

1.5 2.75 4

B

\[ V_{\alpha_{1C}C+R\beta_{2d}} \]

Relative frequency

0 0.2 0.4 0.6 0.8

C

\[ V_{\alpha_{1C}C+R\beta_{3}} \]

Relative frequency

0.2 0.4 0.6 0.8

D

\[ V_{\alpha_{1C}C+R\beta_{2d}} \]

Relative frequency

0 0.25 0.5 1

E

\[ V_{\alpha_{1C}C+R\beta_{2d}} \]

Relative frequency

0 0.1 0.2

F

\[ V_{\alpha_{1C}C+R\beta_{3}} \]

Relative frequency

0 0.1 0.2

Donor/Acceptor ratio  FRET efficiency  Distance (nm)

\[ r_{V-C} = 69\AA \]

\[ r_{V-C} = 67\AA \]

\[ r_{V-C} = 69\AA \]

\[ r_{V-C} = 67\AA \]

\[ r_{V-C} = 78\AA \]

\[ r_{V-C} = 83\AA \]
Table 1. Intra- and intermolecular distances between the Ca$_{1.2}$ calcium channel $\alpha_{1C}$ and $\beta$ subunits measured by three-color FRET microscopy.

| Channel subunits | Measured distances ($r$) | $\beta_1b$ | $\beta_2d$ | $\beta_3$ |
|------------------|--------------------------|------------|------------|------------|
|                  | $r$, Å                    | $r$, Å     | $c$        | $r$, Å     | $c$        |
| $\nu_{1C}C+R\beta$ | $r_{C-V}$                | 68±1 (17)  | 68±2 (13)  | 0.90±0.37  | 69±1 (19)  | 0.60±0.05  |
|                  | $r_{C-V}$                | 72±3 (5)   | 1.27±0.54  | 77±3 (6)   | 1.16±0.17  |
|                  | $r_{V-R}$                | 95±3 (13)  | 99±33 (6)  | 1.70±0.27  | 90±2 (19)  | 0.97±0.20  |
|                  | $r_{V-R}$                | 107±1 (3)  | 2.52±0.17  | 100±2 (15) | 1.72±0.23  |
|                  | $r_{C-C}$                | 85±2 (13)  | 84±2 (13)  | 79±1 (14)  | 0.70±0.10  |
|                  | $r_{C-C}$                | 85±1 (10)  | 1.55±0.07  |            |            |
| $\nu_{1C}C+\alpha_{1CC}+R\beta$ | $r_{C-V}$                | 67±1* (26) | 72±2 (13)  | 79±4 (10)  |
|                  | $r_{V-R}$                | 90±2 (26)  | 90±2 (13)  | 90±5 (10)  |
|                  | $r_{C-C}$                | 78±11 (26) | 86±2 (6)   | 80±4 (8)   |

$^a$P<0.002 vs. $\beta_2$
$^b$P<0.05 vs. $\beta_2$
$^c$P<0.05 vs. $\beta_3$

FRET efficiency between the indicated fluorophores fused to the $\alpha_{1C}$ and $\beta_{1b}$, $\beta_{2d}$ or $\beta_3$ subunits was measured in the plasma membrane of expressing COS1 cells and fitted to a Gaussian function. In cases when the routine curve fit showed two significantly different Gaussian distributions, the corresponding dispersion coefficients $c$ (mean ± SEM) are shown for both distances (see Experimental Procedures). V – mVenus, C- mCerulean, R – tagRFP. Shown values of $r$ are mean ± SEM. Number of cells is shown in parentheses.

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Figure 4. Estimation of distance $r$ between fluorophores fused to the N- and/or C-termini of the $\alpha_{1C}$ subunit. (A–C) Intramolecular FRET recorded with $\nu_{1C}C$. (D–F) Intermolecular FRET recorded with $\nu_{1C}C+\alpha_{1CC}$. Channels were co-expressed in COS1 cells with $\nu_{2d}$ and $R\beta_{1b}$ (A and D), $R\beta_{2d}$ (B and E) or $R\beta_3$ (C and F). Shown are representative of histograms calculated from single exemplary cells for donor/acceptor ratio (left column), FRET efficiency (middle column) and distance (right column). Relative frequency was calculated for total number of pixels in ROI as described in Methods. The red solid line is the best fit to a Gaussian distribution with indicated means for $r_{C-V}$ and $r_{C-V}$.

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Figure 5. Intramolecular vs. intermolecular FRET in $\nu_{1C}C$. The $\nu_{1C}C$ subunit was co-expressed in COS1 cells with $\nu_{2d}$ and $R\beta_{1b}$ (A) or $R\beta_3$ (B). Shown are histograms of donor/acceptor ratio (left column), FRET efficiency (middle column) and distance (right column) determined in the plasma membrane region of two representative COS1 cells. The red solid line is the best fit to a sum of two Gaussian distributions with indicated means (green dotted lines) for intramolecular ($r_{C-V}$) and intermolecular FRET ($r_{C-V}$).

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n = 13) and β3 (79 ± 4 Å, n = 10) are not significantly different from the τC−V values measured under the same conditions with V2α1C.G. With β1b, the intermolecular distance τC−V measured between V2α1C and τC1C was 67 ± 1 Å (n = 26), a value not significantly different from the estimate for intramolecular V2α1C distance (τC−C = 68 ± 2 Å, n = 17). This explains why the data obtained in the presence of β1b was best fitted by a single Gaussian distribution. Thus, unlike β3 and β3 in the presence of β1b the inter- and intramolecular distances appear to be similar.

The measurements with a mixture of Vα1C and α1C confirm that Ca1,2 calcium channels containing β1b, β2d or β3 subunits are in close proximity to each other, thus supporting their clustering in the plasma membrane. The distance τC−V between the N- and C-termini of the neighbor α1C subunits depends on the type of Ca1,2. In the presence of β1b, the distance τC−V (67 ± 1 Å) is 1.2 nm smaller (< P< 0.002) than with β3 (79 ± 4 Å), while τC−C estimated in the presence of β3 (72 ± 2 Å) is of an intermediate value. Subsequent measurements of three-color FRET between RfB and the fluorophores of the τC1C subunit added more certainty to this general picture (Figure 6f).

FRET between tagRFP-labeled Ca1,2 and mCerulean/mVenus-labeled α1C

The three Ca1,2 subunits selected for our study vary in molecular mass (β1b, 53.2 kDa; β3, 73.5 kDa; β3, 54.5 kDa) and in the size of the variable N-terminal (V1), central (HOOK) and C-terminal (V2) regions (see Figure 6B). There are large differences between the three Ca1,2 subunits in variable regions on both sides of the AID-binding pocket, which anchors Ca1,2 to the I—I linker of α1C (Figure 6f). In spite of that, the intramolecular distance τR−R between RfB and V2α1C, estimated in all tested three-color FRET combinations, including single- or double-labeled α1C (Vα1C+α1C+C+R), Vα1C+C+R), was not significantly different for all tested Ca1,2 subunits except for RfB1b (see § in Table 1). Although the average distances τR−R between RfB and V2α1C were significantly different for RfB1b (85 ± 2 Å, n = 13) and RfB3 (79 ± 1 Å, n = 14), they were not significantly different between RfB and β1C. A superposition of all three simultaneously measured arrangements between RfB and V2α1C (Figure 6c) illustrates differences in the positions of RfB subunits as reflected by statistically significant differences in τR−R and τC−R (Table 1).

Fitting to a sum of two Gaussian distributions did not reveal the second (intermolecular) component of FRET between V2α1C and RfB1b (Table 1). However, in the case of RfB3, two intermolecular FRET components were clearly observed, one corresponding to the distance τR−R = 100 ± 2 Å (in 15 of 19 cells) and the other corresponding to τR−R = 85 ± 1 Å (in 10 of 14 cells). In the presence of RfBβ3, the latter component was not observed (n = 1), suggesting that the related distance τC−R exceeded 102 Å. However, intermolecular FRET between V2α1C and RfBβ3 was distinctly revealed in 3 out of 8 cells in a range close to the limits of resolution of the method with an estimate of τR−R = 107 ± 1 Å (Table 1). Taken together, FRET measurements between RfB and the labeled tails of V2α1C corroborated data on intermolecular FRET obtained with V2α1C+α1C+C+RfB and demonstrated that (a) calcium channels are in close proximity in the plasma membrane, and (b) both the intra- and intermolecular architecture of Ca1,2 channels depend on the type of Ca1,2β present.

Discussion

Ca1,2 calcium channels initiate Ca2+ signal transduction to many different downstream targets in wide variety of cells. Investigation of factors affecting structural organization of Ca1,2 channels is crucial for better understanding the mechanisms of Ca2+ signaling. The tendency of Ca1,2 channels to form clusters in the plasma membrane of different cell types has been poorly investigated. Here we studied effects of three major Ca1,2 subunits on structural organization of recombinant Ca1,2 channels expressed in COS1 cells. Because untransfected COS1 cells do not express endogenous calcium channels, they lack natural intracellular partners (e.g., cardiac RyR2) in proximity of exogenous Ca1,2 channels that might promote their clustering through “junctional” coupling [45]. However, recombinant Ca1,2 channels expressed in COS1 cells establish functional coupling to CREB-dependent transcriptional activation [46], pointing to a physiologically relevant integration of recombinant Ca1,2 into a naturally occurring signaling cascade with Ca2+/calmodulin-dependent protein kinase II mediating this activity in native cells [47].

TIRF microscopy revealed clusters of recombinant Ca1,2 channels in the plasma membrane of COS1 cells. The size and the plasma membrane density of the clusters significantly depend on the type of Ca1,2β present. This important observation suggests that the type of Ca1,2β present determines the structure of the Ca1,2 clusters. The average cluster size varies from 360 (β1b) to 450 nm2 (β3). Corroborating reasonable dimensions of these values, a mean size of the Ca1,2 cluster with the major cardiac β3 (140 nm2) is within the estimated size range (250–560 nm2) of rat ventricular RyR2 clusters [48].

Relative arrangement of τC1C and Ca1,2 was estimated with nanometer precision using three-color FRET microscopy in live cells with calcium channels in a stable, inactivated state. Our study revealed that in spite of substantial differences in molecular structure (Figure 6B), the intramolecular distance between the τC1C subunit tails does not significantly depend on the type of Ca1,2β present. Relative position of RfB1b, RfBβ3 and RfBβ3 did not differ significantly. This is interesting because, unlike β1b and β3, β3 has a C-terminal β,CED domain, which interacts with the IQ region of the τC1C C-tail [44].

Another important observation is that N- and C-termini of τC1C and N-termini of Ca1,2β subunits of neighbor channels are in close (<120 Å) proximity to each other, which corroborates with the tendency of Ca1,2 to form clusters. Intermolecular distance between the τC1C subunit significantly depends on the type of Ca1,2β and increases from 67 Å in the presence of β1b, to 79 Å with β3. Measurements of FRET between RfB and neighbor V/C-τC1C supported this general picture and showed a significant effect of the type of Ca1,2β present on the relative position of neighbor channels.

Interestingly, freeze-fracture of the surface membrane revealed that distances between Ca1,2 channels trapped in cardiac junctions with RyR2 is variable and, on average, are larger than those identified by FRET [49]. It is known that the cytoskeleton and RyR2 associate with Ca1,2 plasma membrane clusters in heart cells [50]. Thus, one can not exclude that the distance between Ca1,2 channels in clusters in cardiac junctions is affected by RyR2. However, it is not clear whether clustering affects the ability of Ca1,2 channels to initiate Ca2+ signaling and whether every channel is responsive to depolarizing stimuli. In cardiac muscle cells, a single Ca1,2 opening triggers activity of 4—6 RyR2 [51]. The average size of a RyR2 cluster in ventricular myocytes plasma membrane is 250 nm2 (~100 RyR2 molecules) [48] and interaction between Ca1,2 and RyR2 is weaker than that between Ca1,1 and RyR1 in skeletal muscle. Thus, activation of a RyR2 cluster may be mediated by random opening of few Ca1,2 channels in clusters located at a larger distance than that estimated by FRET.
Figure 6. Molecular distances between the N- and C-termini of α1C and the Ca,β-subunit N-tail of β1b, β2d and β3. (A) Schematic representation of Vα1C with Rβ arranged under a vertically sliced α1C. The structures of TagRFP and Ca,β core MAGUK region were drawn based on PDB codes 1uisA [37] and 1t0j [62], respectively. FRET measurements with ECFP-labeled plekstrin homology domain in the inner leaflet of the plasma membrane [40,63] showed that the N terminal tags of both the α1C and Ca,β subunits are located within the 2 χ Förster distance (<100 Å for ECFP/EYFP) from the plasma membrane. (B) Schematic representation of the domain organization of β1b, β2d and β3 aligned in regard to AID-binding guanylate kinase (GK) domain (green). Yellow box indicates the Src homology 3 (SH3) domain, purple the variable HOOK region, and blue the β2CED [44]. Number of amino acids is shown inside boxes. Amino acids involved in AID-binding pocket are marked in GK by three horizontal lines (for details see [62,64,65]). (C) Schematic representation of the results of simultaneous measurements of the molecular distances between three fluorophores shown in panel (A) in Vα1C/C/β2d/Rβ in the presence of Rβ1b (black lines), Rβ3 (gray lines) and Rβ2d (red lines).
Little is known about molecular determinants underlying physiologically important cluster organization of Ca_{v1.2} channels in neurons [52]. It was shown recently that scaffolding proteins (AKAP79/150 and PDZ) participating in organizing plasma membrane signaling complexes in neurons were not responsible for organizing Ca_{v1.2} channel clusters [53]. The involvement of Ca_{v}c in Ca_{v1.2} channel clustering, identified in our study, does not contradict the earlier report that the calmodulin-binding IQ region of Ca_{v}c has a role in Ca_{v}c clustering [19]. Because Ca_{v}βs interact with IQ [23,44], it is possible that both act as concerted determinants in Ca_{v1.2} channel clustering.

In conclusion, our study revealed effects of Ca_{v}β subunits on the structural organization of Ca_{v1.2} calcium channel in the plasma membrane in the absence of “junctional” interactions. It remains to be seen whether the observed differences in the cluster packing and arrangement of Ca_{v1.2} contribute to the observed differences in calcium signaling among the cell types with preferential expression of a certain type of Ca_{v}β [54–56].

**Materials and Methods**

**Labeling α1C subunit with mVenus and/or mCerulean**

To avoid dimerization, only monomeric forms of fluorescent proteins were used. The C-terminus of human Ca_{v}1.2 calcium channel α1C(758) subunit was amplified by PCR with sense 5′-cattgatgattatGTCGACAGCCTGTTGGAAAGG-3′ and antisense 5′-cattatcgggt CGAGGTCTGACGTAAGACCCCCTG-3′ primers. The PCR fragment was cleaved with EcoRI and AgeI and incorporated into an mCerulean-N1 [57] vector cleaved with the same enzymes, and the 5′-EcoRV/NotI-3′ fragment from the resulting plasmid was then incorporated into α1C(777)-pCDNA3 cleaved with Alel and NotI, resulting in the mCerulean-N-α1C-C. The 5′-Ndel/Kpnl-3′ fragment from mVenus-C1 vector [26] was incorporated into α1C(777)-pCDNA3 and mCerulean-N-α1C-C cleaved with the same enzymes to yield mVenus-N-α1C-777-pCDNA3 and mVenus-α1C-mCerulean-N-α1C-777-pCDNA3, respectively, coding for V2α1C and V2α1C-C.

**Labeling of Ca_{v}β subunits with monomeric fluorescent tags**

The cDNA of human β1b and β3 subunits was cloned from a human heart mRNA (Promega) by a nest RT-PCR strategy. For β1b, 5′-GAGGGGAGGGGAGGGCCACTAC-3′ was used as primer for the reverse transcription, sense 5′-GAGGGTCTCCTCTCA- TGGTCCAG-3′ and antisense 5′-CCACTACATGGGATGT- TCTGC-3′ primers were used for the first round PCR, sense 5′- GCCACCATGTGCGAAGAGCCAG-3′ and antisense 5′-CAC- TACATGGCAGTGTCTCCTGCTC-3′ primers were used for the second round PCR. For β3, primer 5′-GGCCCTGTGCGGATGAGTTA- GACA-3′ was used for the reverse transcription, sense 5′-GACCTGGGATGCTAGTATGAGG-3′ and antisense 5′-GGCTGTCAGTAGTATGCTG-3′ primers were used for the first round PCR, sense 5′-GCCACCATGTG- GACGATCT-3′ and antisense 5′-GTTCAGTAGCTATG- CTTGGG-3′ primers were used for the second round PCR. The cDNA was cloned into a TA cloning vector pCR 2.1 (Invitrogen) and confirmed by DNA sequencing. The 5′-EcoRV/ BamHI-3′ fragment of a β1b TA clone was incorporated into the pTagRFP-C vector (Invitrogen, Moscow, Russia), which was cleaved with Xhol, filled in with Klenow and then cleaved with BamHI to generate RFP-β1b (R5B1b). In a similar way the 5′-Xhol/HindIII-3′ fragment of a β3- TA clone was incorporated into the pTagRFP-C vector to generate monomeric R5B3. To prepare RFP-β3a, β3d was amplified by PCR using mVenus-R3βa [44] as template with sense primer 5′-CGGAGATCTATGGTGCCAAAGGGCATGTG-3′ and antisense primer 5′-GGGCTGTCAGTATGGGGATGTAACACAG-3′, and then the PCR product was cleaved with BglII and SalI, and incorporated into the pTagRFP-C vector cleaved with the same enzymes.

**FRET calibration constructs**

CTV, C5V, C9V, CVC and VCV were obtained from Drs. Ikeda and Vogel (NIAAA, NIH). The 5′-Ndel/BsrGI[Klenow-filled-in]-3′ fragments of mVenus-C1 and mCerulean-C1 were cloned into pTagRFP-C, which was cleaved with AgeI, filled in with Klenow and then cleaved with NheI, to make V4R and C4R respectively. The 5′- Ndel/BamHI[Klenow-filled-in]-3′ fragment of pTagRFP-C was cloned into mCerulean-N1, which was cleaved with EcoRI, filled in with Klenow and then cleaved with NheI, to make R5C. To prepare R17V and R17C, the 5′-Ndel/Xhol[Klenow-filled-in]-3′ fragment of pTagRFP-C was cloned, respectively, into mVenus-N1 and mCerulean-N1, which were cleaved with BamHI (Klenow filled in) and NheI. CTV was cleaved with BspEI, and the 0.7 kb fragment was inserted into R17V and R17C to generate RTV and RTC, respectively. mCerulean was amplified by PCR using sense primer 5′-TATATCCGGAGATATCAGTGGAGACGGGCGAGAG-3′ and antisense primer 5′-TATAGAATCTTCTTTTGACCTGTC- CCTAGCGCA-3′. After cleavage with BspEI and EcoRI, the PCR product of mVenus was inserted into pTagRFP-C to yield R5V; the PCR product of mCerulean was inserted into pTagRFP-C and C4R to yield R5C and CRC, respectively. RFP was amplified by PCR with sense primer 5′-TATAGAATCTTCTTTTGACCTGTC- CCTAGCGCA-3′ and antisense primer 5′-ATATG- GTACATATTGTTTGCCAGCTTTGCTAG-3′, cleaved with EcoRI and Kpnl, and incorporated into R5V and R5C cleaved with the same enzymes to yield RVRF and CRC, respectively.

**Imaging**

Images were recorded with a pixel size of ca. 200 nm using a 14-bit Hamamatsu C9100-12 digital camera (Hamamatsu City, Japan) mounted on a Nikon TE2000 epifluorescence microscope (Tokyo, Japan) equipped with a 60×1.45 numerical aperture (n.a.) oil objective and multiple filter sets (Chroma Technology, Rockingham, VT). Excitation light was delivered by a 175 W xenon lamp. Excitation filter sets were changed by a high-speed filter wheel system (Lambda 10-2, Sutter Instrument, Novato, CA). The Dual-View system (Optical Insights, Santa Fe, NM) was used for the simultaneous acquisition of two fluorescence images (donor and FRET). Images were collected and analyzed using C-Imaging (Compx, Cranberry Township, PA) and MATLAB v.7.0.4 (The Mathwork, Natick, MA).

Two-color FRET was quantified with three filter sets for the yellow fluorescent protein (YFP) cube, excitation filter 500/20 nm, dichroic beam splitter 515 nm, emission filter 535/30 nm; for the cyan fluorescent protein (CFP) cube, excitation filter 436/20 nm, dichroic beam splitter 505 nm, emission filter 480/40 nm; for the FRET cube (CFP/YFP), excitation filter 436/20 nm, dichroic beam splitter 505/30 nm, emission filter 540/30 nm. For three-color FRET, the six-filter method described in [39] was used. All FRET images were acquired sequentially. For imaging mCerulean/mVenus pairs, the same filter arrangement as for two-color FRET was used. For the mCerulean/tagRFP combination, the following settings were used: for CFP cube, excitation filter 436/20 nm, dichroic beam splitter 505 nm, emission filter 480/40 nm; for RFP cube, excitation filter 555/28 nm, dichroic beam splitter 565 nm, emission filter 630/50 nm; for the FRET cube, excitation filter 436/20 nm, dichroic beam splitter 565 nm, emission filter 630/50 nm. With the mVenus/tagRFP combination, the following filter arrangement was used: for
YFP cube, excitation filter 500/20 nm, dichroic beam splitter 515 nm, emission filter 535/30 nm; for RFP cube, excitation filter 555/28 nm, dichroic beam splitter 565 nm, emission filter 630/50 nm; for the FRET cube, excitation filter 484/15, dichroic beam splitter 560 nm, emission filter 630/50 nm. TIRF images were obtained with TIRF2 Nikon system mounted on Nikon TE2000 microscope and argon-ion laser with 514 nm line and diode laser with 440 nm line, dichroic beam splitter 505 nm, emission filters 470/30 nm and 550/30 nm.

Clusters within TIRF images were identified using 2D continuous wavelet transform similar to [58]. Images were analyzed using a two-dimensional mexican hat wavelet over scales 0.5 through 2 to identify ROI of locally increased signal fluorescence up to 5 μm² in area. Similar approaches have been employed for cluster detection in clinical and cell biology imaging [46,59,60]. Corrected FRET intensity was calculated from data acquired using the three filter sets (CFP, YFP, and FRET) as described previously [40] using MATLAB. Briefly, corrected FRET values (FRET) were calculated according to

$$FRET_c = I_{FRET} - aI_d - bI_a,$$

where a and b are bleedthrough coefficients and $I_{FRET}$, $I_d$ and $I_a$ are FRET, donor and acceptor intensities.

Measurement of the G factor, which relates the increase in sensitized acceptor emission to the loss of donor fluorescence (quenching), is critical for calculating FRET efficiency (E) using the three-filter cube method. G factor is a constant for a particular fluorophore pair and imaging setup [42]. This method requires preparation of cDNA constructs encoding donor-acceptor fusion fluorescent proteins differing as widely as possible in FRET efficiency. This was accomplished by varying the length and composition of the linker residues connecting mCerulean and mVenus, mCerulean and tagRFP or mVenus and tagRFP. G factor was determined as

$$G = \frac{F_{a1}/I_{aa2} - F_{d2}/I_{dd2}}{I_{aa2}/I_{aa1} - I_{dd2}/I_{dd1}},$$

where $I_{aa2}$, $I_{dd2}$ and $F_{d2}$ are acceptor, donor and corrected FRET intensity of the construct with the shortest linker between donor and acceptor, and $I_{aa1}$, $I_{dd1}$ and $F_{d1}$ are acceptor, donor and corrected FRET intensity of the construct with the longest linker between donor and acceptor. Using this formula, we found G factors of 1.81 for the mCerulean/mVenus pair, 1.30 for the mVenus/tagRFP pair, and 0.38 for the mCerulean/tagRFP pair. These G factor values allowed us to calculate FRET efficiency according to [42] as follows:

$$E = \frac{F_d/G}{I_{dd1} + F_d/G}$$

The distance between two fluorophores was calculated in accordance with Förster theory:

$$r = \sqrt{\frac{R_0^6}{6} \left( 1 - \frac{E}{E} \right)}$$

The Förster distances ($R_{0b}$), the characteristic distance where the FRET efficiency is 50%, was calculated according to [61]:

$$R_0^6 = Ck^2\eta^{-4}Q_D\Delta A(\lambda),$$

where $Q_D$ is the donor quantum yield, $\eta$ is the maximal acceptor extinction coefficient, and $\Delta A(\lambda)$ is the spectral overlap integral between the normalized donor fluorescence and the acceptor excitation spectra. All these parameters were calculated based on data obtained from Evrogen for tagRFP and reported for mCerulean and mVenus in [61].

The $k$ factor for mCerulean/mVenus was calculated to be 0.41, while mVenus/tagRFP gave $k = 1.60$ and mCerulean/tagRFP gave $k = 0.27$.

D/A ratio for arbitrary concentrations of donor and acceptor was calculated according to [42]:

$$D/A = \frac{I_{dd1} + F_d/G}{I_{aa1}k}$$

For corrected FRET efficiency measurements, this ratio should be in the range from 0.2 to 5.0 [41]. During analysis, the pixels with D/A ratio outside this range were eliminated from the FRET efficiency calculations.

Validation of G and k factors is presented in Figure S1 for two- and three-color FRET standards with different FRET efficiencies (linkers) and D/A stoichiometry. In our three-color FRET experiments, the major energy transfer was observed directly between mCerulean and tagRFP and not from cascade transfer through mVenus. If there would be a significant contribution of cascade FRET through mVenus, we would see a decrease in efficiency when we used two-color FRET (mCerulean/tagRFP) compared with three-color FRET, potentially including contributions from mCerulean/mVenus/tagRFP cascade. We did not observe a decrease in efficiency with two-color FRET, as experiments with Rb3 and $\alpha_{AT}$C gave the same efficiency of 0.05 ($r = 80$ nm) as three-color FRET experiments with Rb3, $\alpha_{AT}$C and V$\alpha_{AT}$C. Additional control experiments showed that the third fluorophore did not have a significant effect on mCerulean-mVenus FRET: we did not observe a significant difference between the FRET efficiency of mCerulean/mVenus fluorophores (73 ± 3, n = 10) measured by two-color FRET with V$\alpha_{AT}$C and unlabeled $\beta$3d and that obtained with three-color FRET using Rb3d and V$\alpha_{AT}$C (68 ± 2 nm, n = 13).

For each cell, we calculated FRET efficiency and distances ($r$) between fluorophores in each pixel of ROI Gaussian fitting of the r distribution (20 bin histogram) was done in MATLAB using the fit function:

$$f(r) = ae^{-\left(\frac{r-b}{c}\right)^2},$$

where $b$ is the position of the center of the peak (mean) and $c$ (dispersion coefficient) reflects the width of the distribution.
FRET efficiency and donor/acceptor ratio of FRET standards. Shown are bar graphs summarizing the mean FRET efficiency (A) and the D/A ratio (B) for the indicated FRET calibration standards. Data are presented as mean ± SEM. (A) FRET efficiency values were: C4R (0.110 ± 0.005), R39C (0.081 ± 0.003), CTV (0.023 ± 0.004), V4R (0.433 ± 0.011), RTV (0.191 ± 0.009), C5V (0.474 ± 0.014), C39V (0.266 ± 0.014) and CTV (0.179 ± 0.006). (B) D/A ratios were: C4R (0.99 ± 0.009), R39C (1.00 ± 0.10), RTV (1.00 ± 0.06), CRC (1.96 ± 0.10), RCR (0.48 ± 0.03), V4R (1.00 ± 0.07), RTV (1.00 ± 0.10), RVR (0.53 ± 0.09), C5V (1.00 ± 0.04), C39V (0.95 ± 0.09), CTV (1.00 ± 0.06), CVC (2.00 ± 0.10) and VCV (0.54 ± 0.02). The number of tested cells is shown in the bars. As one can see, increasing the length of the linker between the fluorophores significantly reduced FRET efficiency consistent with an increased conductance and open probability of L-type Ca\textsuperscript{2+}-channels. FEBS Lett 477: 169–169.

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