Adrenergic Signaling Controls RGK-Dependent Trafficking of Cardiac Voltage-Gated L-Type Ca\(^{2+}\) Channels Through PKD1

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**Rationale:** The Rad-Gem/Kir-related family (RGKs) consists of small GTP-binding proteins that strongly inhibit the activity of voltage-gated calcium channels. Among RGKs, Rem1 is strongly and specifically expressed in cardiac tissue. However, the physiological role and regulation of RGKs, and Rem1 in particular, are largely unknown.

**Objective:** To determine if Rem1 function is physiologically regulated by adrenergic signaling and thus impacts voltage-gated L-type calcium channel (VLCC) activity in the heart.

**Methods and Results:** We found that activation of protein kinase D1, a protein kinase downstream of α1-adrenergic signaling, leads to direct phosphorylation of Rem1 at Ser18. This results in an increase of the channel activity and plasma membrane expression observed by using a combination of electrophysiology, live cell confocal microscopy, and immunohistochemistry in heterologous expression system and neonatal cardiomyocytes. In addition, we show that stimulation of α1-adrenergic receptor-protein kinase D1-Rem1 signaling increases transverse-tubule VLCC expression that results in increased L-type Ca\(^{2+}\) current density in adult ventricular myocytes.

**Conclusion:** The α1-adrenergic stimulation releases Rem1 inhibition of VLCCs through direct phosphorylation of Rem1 at Ser18 by protein kinase D1, resulting in an increase of the channel activity and transverse-tubule expression. Our results uncover a novel molecular regulatory mechanism of VLCC trafficking and function in the heart and provide the first demonstration of physiological regulation of RGK function. (Circ Res. 2012;110:59-70.)

**Key Words:** adrenoceptor ▪ patch clamp ▪ phenylephrine

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The voltage-gated calcium channels play a crucial role in regulating cellular excitability.1 In cardiac muscle, Ca\(^{2+}\) influx through voltage-gated L-type calcium channels (VLCCs) regulates cardiac rhythm and controls muscle contractility by triggering Ca\(^{2+}\) release from the sarcoplasmic reticulum via excitation-contraction coupling.2-4 The alteration of VLCC density or function at the plasma membrane is a key regulator of Ca\(^{2+}\)-dependent cell signaling, gene expression, and cell growth associated with a variety of cardiac diseases, including heart failure, ischemic heart dysfunction, and cardiac arrhythmias, demonstrating a central role for posttranslational modification of VLCC function in cardiac disease.5-8 The VLCCs in the heart are multisubunit transmembrane proteins (pore-forming α\(_i\)-subunits, also named Cav1.2, and auxiliary subunits α\(_{2,3}\) and β\(_{2,3}\)) that open in response to membrane depolarization.1-4 VLCCs are particularly localized at the sarcolemmal membrane structure called the transverse tubular (T-tubule) system.10 T-tubules occur at the Z-line and at the end of each sarcomere, and they also show complex networks of branching tubules with both transverse and longitudinal elements,10 which have crucial roles for the regulation of cardiac muscle contractility and rhythm.3,4,11,12 Although acute modulation of VLCCs by neurotransmitters such as adrenergic stimulation has been extensively studied,1,4,11-14 surprisingly little is known about the molecular mechanisms underlying dynamic physiological and pathophysiological regulation of VLCC membrane expression by intracellular signal transduction.

Emerging evidence suggests that members of the RGK family (Rem1, Rem2, Rad, and Gem/Kir) strongly inhibit VLCC trafficking and activity when overexpressed in heterologous expression systems or native cells, including heart,
skeletal muscle, and brain. In particular, Rem1, a member of the RGK family, is abundantly expressed in the heart. However, in native cells including cardiomyocytes, the physiological role of RGK proteins and their regulation by intracellular signaling are largely unknown.

Here we show that adrenergic stimulation releases Rem1 inhibition of VLCC. The release of Rem1-mediated VLCC inhibition in adult cardiomyocytes dramatically increases both T-tubule VLCC membrane expression and Ca\(^{2+}\) current density. We further show that adrenergic stimulation release of Rem1 inhibition of VLCC results from activation of protein kinase D1 (PKD1), a protein kinase downstream of Rem1 inhibition of VLCCs (IC\(_{a}\)) because of increased VLCC plasma membrane expression. These findings uncover a novel molecular mechanism that modulates VLCC trafficking and function and provide the first demonstration of physiological regulation of RGK function.

### Materials and Methods

An expanded Methods section is available in the Online Data Supplement (http://circres.ahajournals.org/).

#### Plasmid, Antibodies, and Reagents

All plasmids, antibodies, and reagents used for the experiments are shown in the Online Data Supplement. Antiphospho-Rem1 (Ser18) was generated with a synthetic phosphopeptide corresponding to mouse Rem1 residues 12 to 24.

#### Cell Culture, Transfection, and Infection

HEK293T cells and Hela cells were transfected with plasmids and used for experiments 24 hours after transfection. Neonatal and adult rat ventricular myocytes were isolated, cultured, and infected with recombinant adenoviruses as previously described.

#### In Vitro Kinase Assays

Glutathione S-transferase fusion protein expression plasmids for full-length wild-type (WT-Rem1) and Rem1 mutants were generated and used for in vitro kinase assays for PKD1.

#### Biochemistry

Whole-cell lysates were used for Western blot and immunoprecipitation analyses. The expression level of Cav1.2 in the plasma membrane was determined by a cell-surface protein biotinylation assay.

### Confocal Microscopy

Plasma membrane localization of Cav1.2 was quantified by line scan intensity measurements and reported as membrane/cytosol ratio (M/C ratio).

### Electrophysiology

Whole-cell patch-clamp experiments were conducted to measure IC\(_{a}\) at room temperature (\(\sim22^\circ\)C) using extracellular solution containing 10 or 1 mmol/L Ca\(^{2+}\) in HEK293T cells and cardiomyocytes, respectively.

### Data and Statistical Analyses

All results are shown as mean±SE. The number of the cells used for each analysis is shown in parentheses in the graphs. Unpaired Student t tests were performed when comparing two data sets. For multiple comparisons, a one-way ANOVA followed by post hoc Tukey test were performed. Statistical significance was set as \(P<0.05\).

### Results

#### α\(_{1}\)-AR Stimulation Attenuates the Inhibitory Effect of Rem1 on VLCC Function and Plasma Membrane Expression

Rem1 is expressed in cardiomyocytes but is not endogenously expressed in HEK293T cells. To explore whether adrenergic signaling can release the inhibitory effect of Rem1 on IC\(_{a}\), we coexpressed VLCC subunits with Rem1 and ARs (α\(_{1}\)-AR or β\(_{1}\)-AR) in HEK293T cells and determined the subcellular VLCC localization using confocal microscopy. Cav1.2 (pore-forming α-subunit), β\(_{2a}\), and α\(_{28}\) subunits were cotransfected. Cotransfection of all three subunits resulted in the distinct expression of GFP-tagged Cav1.2 in the surface membrane (Figure 1A, B, online Figure II). As previously reported, without coexpression of β\(_{2a}\) subunits, Cav1.2 was not expressed at the plasma membrane (online Figure II). In addition, coexpression of α\(_{28}\) subunits increased the surface membrane expression level of Cav1.2-β\(_{2a}\) channels.

Rem1 coexpression caused Cav1.2 to be largely retained at the endoplasmic reticulum (ER) (Figure 1A, B, Figure 2A, B). Remarkably, the inhibitory effect of Rem1 on VLCC surface expression was dramatically attenuated by α\(_{1}\)-AR stimulation (10 μmol/L phenylephrine [Phe] for 2 hours; Figure 1A, B), concomitant with Cav1.2 redistribution from the ER to the plasma membrane (Figure 2A, B). We determined the dose-dependence of 2 hours of Phe treatment on Cav1.2 expression and found that 0.1 μmol/L Phe significantly increased channel membrane expression, with a maximal effect at 10 μmol/L (online Figure III). The increase in VLCC surface expression by Phe was blocked by the α\(_{1}\)-AR antagonist prazosin (1 μmol/L), confirming that the effect is mediated through α\(_{1}\)-ARs (M/C ratio of Phe-treated=0.93±0.29, \(n=13\); untreated=0.81±0.16, \(n=35\); \(P=0.71\)). Acute α\(_{1}\)-AR stimulation (30 seconds–15 minutes) did not significantly alter VLCC localization, but VLCCs gradually redistributed to the surface membrane after 1 hour of stimulation (online Figure VI). In the absence of Rem1 expression, VLCC membrane expression was not enhanced by Phe stimulation (online Figure II). Rem1-mediated reduction in Cav1.2 surface expression and relief by α\(_{1}\)-AR

Non-standard Abbreviations and Acronyms

| Acronym | Definition |
|---------|------------|
| AR      | adrenoceptor |
| I\(_{\text{ca}}\) | the Ca\(^{2+}\) current through VLCCs |
| M/C ratio | membrane/cytosol ratio |
| Phe | phenylephrine |
| PKD-SE | constitutive active PKD1 |
| PKD1 | protein kinase D1 |
| RGK | Rad-Gem/Kir-related family |
| T-tubules | transverse tubules |
| VLCC | voltage-gated L-type calcium channels |
stimulation were also confirmed by a cell-surface protein biotinylation assay\(^2\) (online Figure V).

VLCCs function was estimated in whole-cell patch experiments. Consistent with reduction in surface membrane expression, Rem1 expression markedly decreased IC\(_{\text{a}}\) as previously reported for Rem1 and other RGKs.\(^{15}\) The 1-AR stimulation (10 \(\mu\)mol/L phenylephrine (Phe) for 2 hours) restored IC\(_{\text{a}}\) magnitude to levels comparable to that observed in the absence of Rem1 without altering the voltage dependence of channel activation (Figure 1D). Acute activation of 1-AR signaling did not activate IC\(_{\text{a}}\) both in the presence and absence of Rem1 in this cell line (online Figure VI). To functionally assess the plasma membrane expression level of the channels under these conditions, we activated channels with the Ca\(^{2+}\) channel agonist Bay K 8644.\(^{21}\) In control cells, IC\(_{\text{a}}\) was significantly increased (online Figure VII) as previously reported\(^{21}\) by Bay K 8644 treatment. In Rem1-transfected cells, IC\(_{\text{a}}\) was also significantly increased by Bay K 8644 treatment. However, the average fold increase in IC\(_{\text{a}}\) is the same among these three groups and IC\(_{\text{a}}\) in Rem1-transfected cells still remained at lower levels than those observed in the absence of Rem1 or in the presence of Rem1 after Phe stimulation (online Figure VII). These results indicate that IC\(_{\text{a}}\) inhibition by Rem1 under these conditions is mainly attributable to a decrease in VLCC plasma membrane expression, which can be released by 1-AR stimulation.

To explore whether 1-adrenergic signaling can also release the inhibitory effects of Rem1 on IC\(_{\text{a}}\), we coexpressed VLCC subunits with Rem1 and 1-ARs in HEK293T cells. However, the inhibitory effect of Rem1 on VLCC trafficking was not reversed by 1-AR stimulation (100 nmol/L isoproterenol for 2 hours) in these experiments (online Figure VIII). Because 1-AR shows agonist-induced internalization during long-term agonist stimulation, not observed with 1A-AR (online Figure IX), we also used a direct adenylyl cyclase activator (1 \(\mu\)mol/L forskolin for 2 hours) to directly activate downstream 1-AR signaling. The inhibitory effect of Rem1 on VLCC trafficking also was not reversed by forskolin applications (online Figure VIII), indicating that release of Rem1 inhibition on IC\(_{\text{a}}\) is specific to 1-AR signaling.

**Figure 1.** The 1-adrenoceptor (AR) stimulation attenuates the inhibitory effect of Rem1 on voltage-gated L-type calcium channel (VLCC) function and surface-membrane expression. A, Subcellular localization of GFP-tagged Cav1.2. VLCC subunits and 1A-AR were cotransfected with (middle, right) or without (left) wild-type (WT)-Rem1 in HEK293T cells. Rem1-transfected cells were also stimulated with 10 \(\mu\)mol/L phenylephrine (Phe) for 2 hours (right). GFP-emission profiles at a cross-section of the cells are shown. AU indicates fluorescence arbitrary units. B, Effect of Rem1 expression and Phe stimulation on VLCC localization. The ratio of fluorescence intensity at the surface membrane and cytosol was shown as membrane/cytosol ratio (M/C ratio; online Figure XXVII). The number of the cells used for each condition is shown in parentheses. NS indicates not significant. C, Effect of Rem1 expression and Phe stimulation on the Ca\(^{2+}\) current through VLCC (IC\(_{\text{a}}\)). Representative family of IC\(_{\text{a}}\) traces are obtained from the cells shown in (A). D, Effect of Rem1 expression and Phe stimulation on current–voltage relationship of IC\(_{\text{a}}\).
the cardiovascular system.18,19 Ser18 of Rem1 lies within a PKD consensus motif LXRXX(T*/S*) (Figure 3A) conserved across multiple eukaryotic Rem1 species (online Figure X). The amino acid sequence surrounding Ser 290 of Rem1 also is closely related to the PKD consensus motif (Figure 3A). To examine whether PKD1 could phosphorylate Rem1 at either of these potential sites, we performed in vitro kinase assays using glutathione S-transferase-fusion proteins and found that Ser18 (but not Ser290) is a PKD1-specific phosphorylation site in Rem1 (Figure 3B). We further demonstrated that PKD1 phosphorylates Rem1-Ser18 in situ (Figure 3C) using a custom-made antibody (online Figure XI). Moreover, PKD1 interacted with Rem1, and this interaction increased when PKD1 was activated (online Figure XII). To test whether α1-AR signaling phosphorylates Rem1-Ser18 through PKD1 activation, we cotransfected α1-AR and Rem1 into HEK293T cells and stimulated the cells with phenylephrine. In unstimulated cells, a low-level Rem1 phosphorylation was observed, suggesting that PKD signaling has some activity under basal conditions (Figure 3D). Phosphorylation of Rem1-Ser18 was increased within 30 seconds of Phe stimulation, concomitant with endogenous PKD1 activation, and this effect remained for up to 2 hours (Figure 3D, online Figure XIII). Rem1 phosphorylation was also observed with stimulation by lower concentrations of Phe (0.1 μmol/L; online Figure XIV). Increased Rem1-Ser18 phosphorylation by Phe was blocked by the pretreatment with 1 μmol/L prazosin (online Figure XV). In addition, PKD1 activation, as observed after α1-AR stimulation, promoted Cav1.2 redistribution from the ER to the surface membrane (Figures 2, C and D, and 4A). Expression of the Rem1-Ser18Ala mutant (Rem1SA) abolished the α1-AR-mediated and PKD1-mediated regulation of ICa and Cav1.2 trafficking to the surface membrane (Figure 4, B and C). Similarly, coexpression of a kinase-negative PKD1 mutant reduced the phosphorylation of Rem1 and abolished rescue of ICa produced by Phe (online Figure XVI). Results from direct cell application of a PKC activator and cAMP analog demonstrated that PKD1 activation and Rem1 phosphorylation occurs downstream of PKC but not cAMP (online Figure VIII). Collectively, these results indicated that PKD1 directly phosphorylates Rem1-Ser18 on α1-AR stimulation, promoting VLCC plasma membrane localization and, thus, increasing ICa.

Rem1-Ser18 is a potential phosphorylation site suggested to be required for the binding RGKs to the scaffolding protein 14-3-3 in vitro, which is thought to regulate subcellular RGK localization.15 However, the upstream signaling pathway that controls RGK phosphorylation...
remains unknown. We found that Ser18 phosphorylation promoted Rem1 binding to 14-3-3 (online Figure XVII) and translocation to the nucleus (online Figure XVIII), suggesting that Ser18 phosphorylation increases the ability of 14-3-3 to recruit Rem1, thereby interfering with the ability of Rem1 to associate with VLCC and inhibit VLCC surface membrane trafficking and function. Rem1 in unstimulated cells strongly colocalized with the ER marker, consistent with the role of Rem1 in suppressing VLCC membrane expression.

Figure 3. The α1-adrenoceptor (AR) stimulation triggers protein kinase D1 (PKD1)-mediated phosphorylation of Rem1 at Ser18. 
A, Diagram highlighting two potential PKD motifs in Rem1. B, In vitro phosphorylation of Rem1 and mutant Rem1 glutathione S-transferase-fusion proteins (upper). Equal loading was verified by Ponceus S staining of membrane (lower). Bar graphs (right) show the summary data (n=3). C, Constitutive active PKD1 (PKDSE) phosphorylates Rem1-Ser18 in Hela cells. D, The α1-AR stimulation activates PKD1 and induces Rem1 phosphorylation at Ser18 in HEK293T cells (n=3).
Figure 4. The α1-adrenergic receptor (AR) stimulation regulates voltage-gated L-type calcium channel (VLCC) surface membrane expression through protein kinase D1 (PKD1)-dependent phosphorylation of Rem1 at Ser18. A, Constitutively active PKD1 (PKD-SE) induces VLCC membrane expression in HEK293T cells cotransfected with Rem1. VLCC subunits and Rem1 were cotransfected with or without PKD-SE. Representative confocal image of cells transfected with VLCC, Rem1, and PKD-SE expression results in GFP-tagged Cav1.2 primarily localized in the surface membrane (left). The membrane/cytosol ratio (M/C ratio) of fluorescence intensity is shown compared with control cells transfected with VLCC subunits (middle). Current-voltage relationships were obtained from these three groups (right).

B, Mutation of the PKD phosphorylation site in Rem1 (S18A) attenuated PKD1-induced VLCC expression at the plasma membrane. VLCC subunits and mutant Rem1-S18A were cotransfected with or without PKD-SE. Representative confocal image from a cell cotransfected with VLCC, Rem1-S18A, and PKD-SE shows that GFP-CaV1.2 subunits were primarily localized within the cytosolic region of the cell (left). The M/C ratio of fluorescence intensity compared with control cells (middle). Current-voltage relationships were obtained from these three groups (right).

C, Expression of mutant Rem1-S18A blocked phenylephrine (Phe)-induced enhancement of Ca²⁺ channel expression in the plasma membrane. VLCC subunits, α1-AR, and mutant Rem1-S18A were cotransfected in HEK293T cells. Cells were treated with or without Phe for 2 hours. Representative confocal image of cells transfected with VLCC subunits, α1-AR, and Rem1-S18A shows that GFP-CaV1.2 subunits were primarily localized in the surface membrane after Phe stimulation (left). The M/C ratio of fluorescence intensity is shown compared with control cells (middle). Current-voltage relationships were obtained from these three groups (right).
**α₁-AR Stimulation Enhances VLCC Expression at the Plasma Membrane Through PKD1-Dependent Phosphorylation of Rem1 at Ser18 in Neonatal Cardiomyocytes**

Our results indicate that α₁-AR stimulation results in PKD1-mediated phosphorylation of Rem1 at Ser18 and a subsequent increase in VLCC surface membrane expression and function after heterologous expression in HEK293T cells. Next, we investigated the role of the proposed α₁-AR-PKD1-Rem1-VLCC signaling pathway in native cardiomyocytes. We found that Rem1 is expressed in whole-cell lysates of neonatal rat ventricular myocytes and that α₁-AR stimulation by Phe (30 minutes) promoted PKD1 activation and Rem1 phosphorylation at Ser18 (online Figure XIX). In addition, we determined the subcellular localization of VLCC in neonatal cardiomyocytes before and after α₁-AR stimulation by Phe using an anti-Cav1.2 antibody (Figure 5, online Figure XX). In agreement with previous reports, Cav1.2 labeling was observed both at the surface membrane and intracellularly under basal conditions (online Figure XX). After α₁-AR stimulation (10 μmol/L Phe for 2 hours), Cav1.2 was preferentially localized at the plasma membrane with additional nuclear punctuate staining (Figure 5A, online Figure XX), presumably because of the stimulation of endogenous Rem1. The M/C ratio was significantly increased by Phe stimulation (Figure 5A, C, online Figure XX). To confirm the involvement of PKD1 activity in this effect, myocytes were infected with GFP-tagged kinase-negative PKD and cellular localization of Cav1.2 was determined before and after α₁-AR stimulation. GFP infection alone did not alter Cav1.2 localization either before or after Phe treatment (compare with Figure 5A, online Figure XX). However, kinase-negative PKD-infected myocytes did not show a significant increase in Cav1.2 plasma membrane expression in response to Phe (Figure 5B, C). These results demonstrate that α₁-AR stimulation induces an increase of VLCC surface membrane expression through a PKD1-dependent mechanism in neonatal cardiomyocytes. To confirm the involvement of Rem1 phosphorylation at Ser18 in this process, myc-tagged WT-Rem1 or Rem1-S18A was overexpressed by adenoviral infection and the subcellular localization of Cav1.2 was assessed before and after α₁-AR stimulation (Figure 5D–F). Overexpression of both WT-Rem1 (M/C ratio=0.93±0.15, n=9) and Rem1-S18A (M/C ratio=0.88±0.09, n=10) significantly decreased the M/C ratio compared to control (LacZ-infected cells, M/C ratio=1.63±0.24, n=11; P=0.03 and 0.01, respectively). In WT-Rem1-expressing cells, Phe stimulation promoted Cav1.2 redistribution to the plasma membrane and significantly reduced the degree of colocalization with Rem1 (Figure 5D, F, online Figure XXI). However, in Rem1-S18A–infected myocytes, Phe stimulation did not alter Cav1.2 subcellular localization or Rem1-Cav1.2 colocalization (Figure 5E, F, online Figure XXI). These results indicate that α₁-AR stimulation increases VLCC membrane expression in neonatal cardiomyocytes through PKD1-dependent phosphorylation of Rem1 at Ser18.

**α₁-AR Stimulation Enhances T-Tubule VLCC Expression in Adult Ventricular Myocytes Through PKD1-Dependent Phosphorylation of Rem1 at Ser18**

We next investigated the role of the α₁-AR-PKD1-Rem1-VLCC signaling pathway in the heart by measuring Cav1.2 localization and ICa function in adult rat ventricular myocytes in response to sustained α₁-AR stimulation. By using the plasma membrane marker wheat germ agglutinin, we confirmed that the cellular morphology, T-tubule structure, and its periodicity (≈1.8 μm)were preserved in our cultured myocytes up to 40 hours after infection (Figure 6A, D, online Figures XXII, XXIII).

We measured the effect of Rem1 and Rem1(S18A) overexpression and α₁-AR stimulation on Cav1.2 localization and ICa in adult cardiomyocytes. We used fast fourier transform power spectral analysis of Cav1.2 immunofluorescence to quantify VLCC T-tubule localization. In WT-Rem1–overexpressing myocytes, α₁-AR stimulation promoted Rem1 phosphorylation, Cav1.2 T-tubule redistribution, and partially recovered ICa without changes in Cav1.2 protein expression (Figure 6A–C, online Figure XXV). Rem1 colocalized well with T-tubule Cav1.2 channels before, but not after, Phe stimulation (online Figure XXVI). In Rem1-S18A–overexpressing adult ventricular myocytes, α₁-AR–mediated regulation of VLCC T-tubule expression and ICa activation were not observed (Figure 6D–F). These results indicate that α₁-AR stimulation increases both VLCC function and T-tubule localization in adult cardiomyocytes through PKD-dependent phosphorylation of Rem1 at Ser18. All experiments were measured 40 hours after Rem1 adenovirus infection; the effects of Rem1 overexpression on channel membrane localization were not observed 24 hours after infection with WT-Rem1 adenovirus (online Figure XXV), although cells expressed four-times to five-times more Rem1 compared to endogenous Rem1 expression, consistent with the slow turnover observed for the Cav1.2 protein. Forty hours after infection with WT-Rem1 adenovirus, myocytes expressed eight-times to 10-times more Rem1 compared to endogenous Rem1 (online Figure XXV).

To determine whether PKD1 activation could regulate VLCC without overexpression of Rem1, presumably by regulating endogenous Rem1, we measure the effect of α₁-AR stimulation (10 μmol/L Phe for 2 hours) on VLCC T-tubular distribution and current. Phe treatment increased ICa in both freshly isolated and cultured adult ventricular myocytes (online Figures XXIII, XXIV). Cultured cardiomyocytes were infected with Lac-Z as control for Rem1-infected cells. VLCC T-tubular distribution was increased after Phe application without changing the total Cav1.2 expression levels (online Figures XXIII, XXV). T-tubular redistribution of Cav1.2 induced by α₁-AR stimulation was abolished by infection with kinase-negative PKD (online Figure XXVI).

**Discussion**

In the present study, we characterize a novel molecular mechanism for the regulation of VLCC cell-surface expression. We show that PKD1 induces an increase in
cell-surface VLCC density through phosphorylation of small GTP-binding protein Rem1 in response to $\alpha_1$-AR stimulation, leading to a subsequent increase in Ca$^{2+}$ channel activity (Figure 7). Our study demonstrates that Rem1 is a PKD1 substrate and that a novel $\alpha_1$-AR-PKD1-Rem1 signaling pathway dynamically regulates VLCC function in cardiomyocytes. In particular, in adult ventricular myocytes, adrenergic stimulation releases Rem1 inhibition of VLCCs, resulting in an increase in channel activity and expression at T-tubules. T-tubule localization...
of VLCCs is key to the control cardiac excitability and contractility. Our results uncover a novel molecular regulatory mechanism of VLCC trafficking and function, and provide the first demonstration of physiological regulation of RGK function.

Previous reports proposed that RGK-mediated Ca²⁺ current suppression in heterologous expression systems results from either a decrease in the number of pore subunits of Ca²⁺ channels expressed at the plasma membrane or an inhibition of surface membrane channel activity. Our data agree with Rem1 decreasing VLCC expression at plasma membrane in both a heterologous expression system and in native cardiomyocytes. Moreover, changes in VLCC membrane localization correlated well with the functional effects observed. Our data suggest that whereas short-term expression of Rem1 may inhibit channel activity without decreasing membrane expression, longer-term Rem1 expression leads to a decrease in channel membrane levels. This is consistent with the slow turnover observed for this channel and suggests that Rem1 may decrease channel insertion into the plasma membrane. Interestingly, the PKD1-Rem1 (S18)-mediated increase in membrane expression of VLCC is observed after only 1 hour of persistent activation of the signaling, suggesting that PKD1-Rem1 signaling can release a VLCC reserve that would help maintain VLCC activity at persistently high adrenergic states. Taken together, our data indicate channel trafficking to be a major contributor to PKD1-Rem1 regulation of VLCC in cardiomyocytes.

Our data would also suggest that the increase in VLCC membrane expression through α₁-AR-PKD1-Rem1 signaling could contribute to the cytosolic Ca²⁺ overload when catechol-
amine levels are chronically and strongly increased under pathophysiological stress conditions, such as cardiac hypertrophy and heart failure. However, potential limitations of the present study include the alteration in integrity of plasma and intracellular membrane systems such as T-tubules and ER membranes in isolated cardiomyocytes after culture and after 2 hours of Phe treatment. Further investigation would be needed to clarify the detailed mechanism and the role of endogenous expression levels of Rem1 in the regulation of VLCC membrane expression by $\alpha_{1}$-AR-PKD1 signaling in cardiomyocytes and in vivo under the physiological conditions.

Among RGKs, both Rem1 and Rad are expressed at significant mRNA and protein levels in the heart and are thought to regulate VLCC function. Although Ser18 is conserved in both proteins, it is not part of a PKD1 substrate motif in Rad. However, further investigation is needed to clarify the involvement of Rad in the regulation of VLCC by $\alpha_{1}$-AR-PKD1 signaling in cardiomyocytes. Changes in ER morphology in Rem1-overexpressing cells were observed (Figures 1, 2), possibly because of the Rem1 regulation of cytoskeleton dynamics, as has been reported for other RGKs. Although morphological remodeling of the ER by Rem1 might contribute to Rem1-mediated inhibition of VLCC trafficking, further studies are needed to more precisely define the underlying mechanism.

In conclusion, we provide the first evidence to our knowledge for a receptor-mediated signaling pathway that can dynamically regulate Rem1 inhibition of VLCCs (Figure 7) in the cardiovascular system. Specifically, we show that $\alpha_{1}$-AR-PKD1–mediated phosphorylation of Rem1-S18 dramatically attenuates Rem1 suppression of VLCC membrane expression and function by promoting the association of Rem1 with 14-3-3 and, consequently, by reducing the colocalization of Rem1 and VLCCs. Because alterations in VLCC T-tubular membrane expression and function are implicated in cardiovascular disease, the PKD1-Rem1-VLCCs regulatory pathway will provide new insight into understanding cardiac excitation–contraction coupling regulation and also will provide new therapeutic perspectives for cardiac hypertrophy, heart failure, and arrhythmias.

Figure 7. Proposed $\alpha_{1}$-adrenoceptor (AR)-protein kinase D1 (PKD1)-Rem1-14 to 3-3 mechanism for the regulation of voltage-gated L-type calcium channel (VLCC) membrane expression. 1, Rem1 blocks VLCC membrane expression and inhibits VLCC activity by retaining VLCCs in endoplasmic reticulum (ER). 2, The $\alpha_{1}$-AR stimulation activates PKD1, which then directly phosphorylates Rem1-Ser18. 3, Phosphorylation of Rem1-S18 increases binding to 14-3-3 and induces Rem1 translocation from cytosol to the nucleus. 4, Rem1 nuclear translocation attenuates the inhibitory effect of Rem1 on VLCC expression, releasing the channel from the ER to traffic to the plasma membrane.
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Disclosures

None.

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Novelty and Significance

What Is Known?
- RGK proteins are strong inhibitors of voltage-gated calcium channels.
- One of the RGKs, Rem1, is highly expressed in heart.
- The physiological role of Rem1 and its upstream regulation is unknown.

What New Information Does This Article Contribute?
- The α-adrenergic receptor stimulation dramatically attenuates Rem1-mediated inhibition of VLCC function and promotes T-tubular localization in cardiomyocytes.
- PKD1-dependent Rem1 phosphorylation of Rem1 (S18) mediates the α-adrenergic regulation of VLCC.
- Stimulation of α-adrenergic-PKD1 and endogenous Rem1 signaling regulates cardiac VLCC channels, demonstrating for the first time a physiological role of Rem1 in the heart.

Despite the widespread expression of small GTP-binding proteins (RGK) and their strong inhibition of VLCC in multiple tissues, the physiological regulation of RGK-mediated signaling remains elusive. In addition, despite the fact that PKD has been implicated in the regulation of diverse biological processes, only a few substrates are known. Here, we show that Rem1 is a novel PKD substrate and that adrenergic stimulation regulates plasma membrane expression of cardiac L-type calcium channels through PKD-dependent Rem1 phosphorylation. This work uncovers a novel molecular mechanism of modulation of VLCC function and provides the first demonstration of physiological regulation of the function of the small GTP-binding protein Rem1.
Adrenergic Signaling Controls RGK-Dependent Trafficking of Cardiac Voltage-Gated L-Type Ca\(^{2+}\) Channels Through PKD1

Bong Sook Jhun, Jin O-Uchi, Weiye Wang, Chang Hoon Ha, Jinjing Zhao, Ji Young Kim, Chelsea Wong, Robert T. Dirksen, Coeli M.B. Lopes and Zheng Gen Jin

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**Adrenergic signaling controls RGK-dependent trafficking of voltage-gated L-type Ca\(^{2+}\) channels.**
Bong Sook Jhun\(^1\)*, Jin O-Uchi\(^1\)*, Weiye Wang\(^1\), Chang Hoon Ha\(^1\), Jinjing Zhao\(^1\), Ji Young Kim\(^1\), Chelsea Wong\(^1\), Robert T. Dirksen\(^2\), Coeli M.B. Lopes\(^1+\),§ and Zheng Gen Jin\(^1+\),§.

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MATERIAL & METHODS

**Plasmid, Antibodies and Reagents**

The following plasmids were used for whole experiments: mouse wild-type (WT) and mutant (Rem1-S18A, -S290A, -S18A/S290A) Myc-tagged Rem1 in pME18S (kindly provided by Dr. Pascal Beguin, Institute of Molecular and Cell Biology, Singapore) (2), Hemagglutinin (HA)-tagged mouse WT Rem1 in pcDNA (kindly provided by Drs. Douglas A. Andres and Robert N. Correll, University of Kentucky College of Medicine) (5), GFP-tagged rabbit Cav1.2 (α1c-subunit) and rat Cavα2δ1 (α2δ-subunit) (kindly provided by Drs. Kurt Beam and Joshua Ohrtman, University of Colorado) (4), human Cavβ2a-N4 in pCMV (β2a-subunit) (kindly provided by Drs. Timothy J. Kamp and Jason D. Foell, University of Wisconsin) (6), human HA-tagged constitutive active PKD1 (PKD-SE) and kinase-negative PKD1 (PKD-KN) (Addgene, Cambridge, MA) (16), HA-tagged human α1A-β-adrenergic receptor in pDoubleTrouble (pDT) (kindly provided by Dr. John Hepler, Emory University School of Medicine) (20), GFP-tagged human α1A- and α1B-β-adrenergic receptor (kindly provided by Dr. Akira Hirasawa, Kyoto University, Japan), mouse Flag-tagged β1-β-adrenergic receptor (kindly provided by Dr. Burns Blaxall, University of Rochester), human Flag-tagged β1-β-adrenergic receptor in pcDNA3.1 (kindly provided by Dr. Robert J Lefkowitz, Duke university), GFP-tagged human β1-β-adrenergic receptor (kindly provided by Dr. Graeme Milligan, University of Glasgow, UK), ER localization vector (the red fluorescent protein DsRed, fused with the ER targeting signal from calreticulin and the ER retention signal, KDEL) (15) (kindly provided by Dr. Dirk J Snyder, University of Antwerp, Belgium), DsRed-MST in pIRES2 (generously provided by Dr. Alfred George, Vanderbilt University) (19) and pEGFP-C1 and -N1 (Clontech Laboratories, Mountain View, CA). PKD-KN was subcloned into pEGFP-N1 vector (9). HA-tagged mouse WT Rem1 was subcloned into pEGFP-C1 vector. Adenovirus encoding GFP-PKD-KN, Myc-tagged WT-Rem1, HA-tagged WT-Rem1 and Myc-tagged Rem1-S18A were generated from pEGFP-PKD-KN, pME18S-Myc-tagged Rem1, pcDNA-HA-tagged Rem1 and pME18S- Myc-tagged Rem1-S18A, respectively, using ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA) (9). Adenovirus encoding β-galactosidase (LacZ) or GFP were used as a control (9).

The following antibodies were used for immunoblot, immunoprecipitation and immuno-histochemistry: anti-PKD1 antibody (Sigma-Aldrich Corporation, St. Louis, MO), anti-phospho-PKD Ser744/748 antibody, anti-phospho-PKD Ser916 antibody (Cell Signaling, Danvers, MA), anti-REM1 antibody, anti-14-3-3-β antibody, anti-HA antibody (Santa Cruz Biotechnology), anti-rabbit Cav1.2 antibody (Alomone Labs Ltd, Jerusalem, Israel) and anti-Myc antibody (Invitogen). Rabbit polyclonal antibodies that specifically recognize phosphorylated Rem1 at Ser18 were commercially generated with a synthetic phosphopeptide.
C-Ahx-TLRRRA(pS)TPLLS-amide corresponding to mouse Rem1 residues 12-24 (GenBank Accession Number NP_033073.1) (21st Century Biochemicals, Inc., Marlboro MA). Specificity of this custom-made antibody was shown in Fig.S5.

All reagents were purchased from Sigma-Aldrich Corporation. (St. Louis, MO) unless otherwise indicated: prazosin hydrochloride (Tokyo Chemical Industry Co., Ltd., Tokyo Japan), phorbol 12-myristate 13-acetate (PMA) (Calbiochem, Darmstadt, Germany), Bay K 8644 (Tocris, Ellisville, MO) and Rhodamine-labeled wheat germ agglutinin (WGA) (Burlingame CA).

**Cell Culture, Transfection and Infection**

HEK293T cells (generously provided from Dr. Keigi Fujiwara, University of Rochester) were transfected with FUGENE-HD transfection reagents (Roche, Mannheim, Germany), re-plated at 1 x 10^4 cells per 35-mm dish 6 hours after transfection by using Acutase (Innovative Cell technologies. Inc., San Diego CA) and used for experiments 24 hours after transfection. Equimolar ratio of GFP-Cav1.2, β2a- and α2δ-subunits, α1-AR and Rem1 was transfected. Transfection efficiency of GFP-tagged Cav1.2 and EGFP-Rem1 when expressed alone were high with ≥80% of the cells showing green fluorescence under confocal microscope. Hela cells (generously provided from Dr. Keigi Fujiwara, University of Rochester) were transfected with Lipofectomin 2000 transfection reagent (Invitrogen) and used for experiments 24 hours after transfection.

Neonatal rat ventricular myocytes were isolated from 3- to 4-day-old Sprague-Dawley rats using collagenase type 2 (Worthington, Lakewood NJ), plated onto laminin (BD Bioscience, San Jose, CA)-coated dishes and cultured overnight in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum as described previously (1,8). Seventy two hours after isolation, neonatal cardiomyocytes were infected with recombinant adenoviruses at the indicated multiplicity of infection (m.o.i.) for 24 hours and used for experiments.

Adult rat single ventricular myocytes were prepared from adult male Sprague-Dawley rats (300-400 g) using collagenase type 2 (Worthington, Lakewood NJ) and protease as we previously described (13,17,18) and suspended in Tyrode’s solution (mM): NaCl, 136.9; KCl, 5.4; CaCl_2, 1; MgCl_2, 0.5; NaH_2PO_4, 0.33; HEPES, 5; glucose, 5, pH 7.40 adjusted with NaOH. Myocytes were plated on laminin-coated coverslips and incubated at 37 °C, 5% CO_2 in DMEM containing 5% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 5 mM taurine, 5 mM carnitine, and 5 mM creatine (3,12,14,23,24). After a 2-hr incubation, culture medium was replaced with adenovirus-diluted serum free DMEM and incubated for 2 hr. Serum-free culture medium was changed every day to remove unattached cells. The cells after 24hr and 40hr infection were used for experiments.
**In Vitro Kinase Assays**

To generate Glutathione S-transferase (GST)-fusion protein expression plasmids, full-length WT Rem1 and Rem1 mutants were amplified by PCR with the following primers:

Rem1 Forward 5’-GCTTGGATCCGGATGACTCTTAACACCCAGCAGGA-3’, Rem1 Reverse 5’-GAAAGCGGCGCTCAGAGCACAGCCAGGTTG-3’.

The amplified products were digested with BamHI/NotI and subcloned into pGEX-5X-2 vector. GST-fusion proteins were expressed in Escherichia coli BL21 by induction with isopropyl-1-thio-β-D-galactopyranoside (IPTG) and purified from cleared cellular lysates using gluthathione-sepharose beads (GE Healthcare UK Ltd., Buckinghamshire, England). Equal amounts of GST-WT-Rem1 and Rem1 mutants were used for in vitro kinase assays.

In vitro kinase assays for PKD1 were performed as described previously(10). Briefly, 0.1 μg of recombinant GST-PKD (Calbiochem, Darmstadt, Germany) and 2 μg of purified GST–WT-Rem1 and Rem1 mutants fusion protein substrates were incubated in a reaction buffer containing 4 mM MOPS (pH 7.2), 4 mM MgCl₂, 2 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 50 μM DTT, 100 μM ATP and 10 μCi of [γ-³²P] ATP. The mixture was incubated at 30°C for 30 min, stopped by adding SDS-sample buffer, and electrophoresed in a denaturing polyacrylamide gel. The gel was transferred to Amersham Hybond-P membrane (GE Healthcare), and subjected to autoradiography to visualize radiolabeled protein bands.

**Western Blot Analysis**

Whole cell lysates are prepared from HEK293T cells, Hela cells and cardiomyocytes(7). Cells were washed with cold PBS and then incubated in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.2 % SDS, 2.5 mM sodium pyrophosphate, 1 μM β-Glycerolphosphpate, 1 mM NaVO₄, 50 mM NaF, 0.5% protease inhibitor cocktail on ice for 30 min. Cell lysates were centrifuged at 20,000g for 15 min at 4 °C and the supernatants were collected. The protein concentration was determined using the the BCA method (Thermo Scientific Inc., Rockford, IL) or Bradford method (BioRad, Hercules, California). Total cell lysates were separated by SDS-PAGE and were transferred to nitrocellulose membrane and incubated with primary antibodies, followed by incubating with fluorescence-conjugated secondary antibodies (Invitrogen). Immunoreactive bands were visualized by Odyssey Infrared Imaging System (LI-COR Biotechnology, Nebraska). Densitometric analyses of immunoblots were performed with NIH Image J software. Results were normalized by arbitrarily setting the densitometry of the control sample.

**Immunoprecipitation**
Immunoprecipitation was performed as previously described (7,18). The lysates were incubated with primary antibodies for overnight at 4°C and then protein A/G Plus Agarose (Santa Cruz Biotechnology, Santa Cruz, California) were added for 2 h at 4°C. Immunocomplexes were washed three times with lysis buffer and subsequently subjected to Western blot analysis.

**Cell Surface Protein Isolation**
The expression level of Ca$^{2+}$ channels in the surface membrane was determined by isolating cell surface proteins by Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific Inc., Rockford, IL)(11). One day after the transfection, HEK293T cells were washed with cold PBS, followed by incubation with Sulfo-NHS-SS-Biotin on ice. The biotinylation reaction was quenched by Tris solution. The cells were lysed and the protein concentrations were measured. For each sample, some parts of whole cell lysates were saved as direct input for immunoblot experiments. Isolation of labeled proteins was performed by incubating with NeutrAvidin Agarose beads.

**Confocal Microscopy**
Localization of $\alpha_{1C}$-subunits of VLCC (Ca$_{V}$1.2), Rem1 and adrenoceptors were observed using laser scanning confocal microscope with images obtained using Fluoview software (Olympus, Tokyo Japan). Cardiomyocytes were fixed with 3.7 % paraformaldehyde in distilled water at 4°C for 10 min, incubated overnight with primary antibodies, followed by secondary antibodies for 1 hr(18). Localization of GFP-tagged Ca$_{V}$1.2 or GFP-tagged Rem1 in HEK293T cells was observed without fixation at room temperature. Plasma membrane localization of GFP-tagged Ca$_{V}$1.2 in HEK293T cells or Ca$_{V}$1.2 stained with anti-Ca$_{V}$1.2 antibody in neonatal cardiomyocytes was quantified by line scan intensity measurements through each cell beginning in the cytosol region (avoiding the nucleus area) and ending at the cell periphery using Image J software(22). Membrane thickness was measured and was used for the determination of the background and cytosol region for the analysis (Online Figure XXVII). The background and cytosol region were set at double length of averaged membrane thickness. After subtraction of background intensity, peak fluorescence intensity at the cell membrane was divided by the mean intensity at in the cytosol region. If there is no significant fluorescence peak at the periphery of cells, the membrane thickness was set using the averaged membrane thickness in all control cells in HEK293T cells (≥20 pixels, n=59) or Phe-stimulated cardiomyocytes (≥25 pixels, n=37) and the averaged fluorescence intensity within this region was used as the substitution of peak fluorescence intensity at the membrane.

T-tubule images from fixed adult ventricular myocytes stained with anti-Cav1.2 were analyzed using Image J software and Origin6.1 software (OriginLab Corporation, Northampton,
MA). First, the profile of the immunofluorescence intensities from a 50 μm x 9.2 μm square region (avoiding the nuclear area) in each cardiomyocyte stained with anti-Cav1.2 was obtained. Fast Fourier Transformation (FFT) was performed and this immunofluorescence profile was converted into the frequency domain using Origin6.1. FFT power has been shown to be able to quantitatively measure the regular organization of T-tubule system (21).

Cytosolic and nuclear localization of GFP-tagged Rem1 in HEK293T cells was quantified by line scan intensity measurements through each cell across the nucleus area using Image J software by calculating the nuclear and cytosolic ratio (N/C ratio) of GFP fluorescence.

ER localization of GFP-tagged Rem1 or CaV1.2 in HEK293T cells was quantified by co-expression of DsRed ER marker. The expression of DsRed itself did not show any inhibition of Ca²⁺ channel trafficking (M/C ratio= 4.49±0.76, n=18. No significant changes compared to that of non-transfected cells. P=0.39) and ER morphology.

Quantitative co-localization analysis was performed using Image J software with an Intensity Correlation Analysis plug-in provided by The Bob and Joan Wright Cell Imaging Facility, Toronto Western Hospital (http://www.uhnres.utoronto.ca/facilities/wcif/fdownload.html). Co-localization was estimated using Pearson’s correlation coefficient and Overlap coefficient according to Manders(26) with ranges between 1 and -1 or 1 and 0, respectively, with the value of 1 representing high co-localization.

**Electrophysiology**

A conventional whole cell patch clamp was carried out to measure Ca²⁺ currents through L-type channels (I_Ca) using an Axon 200B amplifier (Axon Instruments) at room temperature (∼22°C) and filtered by a low-pass Bessel filter at 1 kHz. Equimolar ratio of GFP-Cav1.2, β2a⁻ and α2δ-subunits, α₁-AR and Rem1 was transfected. Membrane-localized GFP-Cav1.2 has weaker fluorescence compared to EGFP and we could not detect fluorescence from GFP-Cav1.2 under the normal fluorescence microscope. For patch clamp experiments, we co-transfected with low amount of EGFP-N1 (0.2 ug) for positive identification of transfected cells by bright fluorescence under normal fluorescence microscope.

The composition of extracellular solution was as follows (mM): NaCl, 136.9; CsCl, 5.4; CaCl₂, 10; MgCl₂, 0.5; Na₂HPO₄, 0.33; HEPES, 5; glucose, 5, pH 7.40 adjusted with NaOH. The Ca²⁺ concentration of extracellular solution for recording from cardiomyocytes was set at 1mM(17,18). The pipette solution was (mmol/L): CsCl, 120; Na₂GTP, 0.1; MgCl₂, 5; HEPES, 5; CaCl₂, 0.02; EGTA, 0.05; Na₂-ATP, 5; K₂-creatine phosphate, 5; pH was adjusted to 7.20 with CsOH (25). For measurement of I_Ca, the holding potential was set at -80 mV and a 400-msec depolarization pulse to 0 mV was applied every 10 sec. The amplitude of current was defined as the difference between the peak current and the residual current at the end of the pulse.
Current-voltage (I-V) relationships were obtained using a series of test pulses between -80 and +60 mV in 10 mV increments (18). A Boltzmann fit ($G = g_{\text{max}}/(1 + \exp[-(V - V_{1/2})/k])$) of I-V relationship was used to determine: 1) the steepness or slope factor ($k$), 2) the voltage that elicits half of the maximal activation ($V_{1/2}$) of activation. The $V_{1/2}$ and $k$ values indicate channel sensitivity to activation by voltage. The cell membrane capacitance was not significantly changed by Rem expression and phenylephrine stimulation in HEK293T cell and cardiomyocytes (Online Table II).

**Data and Statistical Analysis**
All results are shown as mean ± standard error (SE). Unpaired Student's t-test was performed for two data sets. For multiple comparisons, one-way ANOVA followed by posthoc Tukey test was performed. Statistical significance was set as a $P$ value of <0.05.
SUPPLEMENTARY TABLE

**Online Table I.** Fitted parameters for the channel voltage dependence of activation observed from current-voltage relationship curves

|        | Control (n=21) | Rem1+Phenylephrine (n=14) | P value |
|--------|----------------|---------------------------|---------|
| $k$    | 6.10± 0.49     | 5.55± 1.41                | 0.81    |
| $V_{1/2}$ | -10.32± 3.96 mV | -14.64± 5.76 mV          | 0.35    |

The steepness or slope factor ($k$) and the voltage that elicits half of the maximal activation ($V_{1/2}$) of activation which indicate channel sensitivity to activation by voltage were determined from a Boltzmann fit (see detail at Material and Methods). Both $V_{1/2}$ and $k$ were not significantly changed between control- and Rem1-transfected cells after Phe stimulation.

**Online Table II.** Cell capacitance in HEK293T cells and adult cardiomyocytes.

**HEK293T cells**

|                | Control | Rem1 | Rem1 +Phe | Rem1SA | Rem1SA +Phe | Rem1 +PKD-SE | Rem1SA +PKD-SE |
|----------------|---------|------|-----------|--------|-------------|--------------|---------------|
| Cell capacitance (pF) | 21.1±3.30 | 17.8±3.30 | 15.6±1.32 | 19.8±1.26 | 17±3.66 | 20.4±1.43 | 21.8±1.95 |
| Cell number | 114 | 52 | 26 | 29 | 6 | 17 | 17 |
| P value     | 0.52 | 0.44 | 0.85 | 0.78 | 0.92 | 0.92 |

**Cardiomyocytes**

|                | LacZ | LacZ +Phe | Rem1 | Rem1 +Phe | Rem1SA | Rem1SA +Phe |
|----------------|------|-----------|------|-----------|--------|-------------|
| Cell capacitance (pF) | 90.4±9.24 | 102±11.4 | 88.0±15.3 | 81.5±15.3 | 81.8±11.6 | 92.5±16.2 |
| Cell number | 11 | 9 | 8 | 11 | 10 | 8 |
| P value     | 0.68 | 0.89 | 0.55 | 0.56 | 0.75 |

Cell capacitance was measured by whole cell patch clamp. Some groups are stimulated by 10 μmol/L Phe for 2 hr and used for experiments. P -value are shown as the comparison with control cells which are expressed α1A-AR and VLCC in HEK293T cells. In cardiomyocytes, P -value are shown as the comparison with the cells which are infected with LacZ with no Phe stimulation.
SUPPLEMENTARY FIGURE

Online Figure I. Rem1 expression in HEK293T cells
Whole cell lysates were prepared from HEK293T cells, Hela cells and neonatal cardiomyocytes. HEK293T cells were transfected with Myc-tagged Rem1 (and PKD) and Whole cell lysates are prepared from transfected cells. Rem1 was detected by anti-Rem antibody (see Material and Methods).

Online Figure II. Cav1.2 expression in HEK293T cells with accessory subunits in the presence of absence of α1-adrenergic stimulation
HEK293T cells were co-transfected with GFP-tagged Cav1.2 and α1-AR. Cells were also transfected with either β2a and/or α2δ-subunits. 24-hr after transfection, cells GFP-tagged Cav1.2 plasma membrane localization was observed by confocal microscope. Some cells were observed after 2-hr stimulation by 10 μmol/L phenylephrine. Membrane / cytosol (M / C) fluorescence ration was used for evaluation (see Material and Methods). "M/C ratio stands for no obvious plasma membrane localization (scattered line). "p<0.05, compared to the cells co-transfected with all 3-subunits before Phe stimulation.
Online Figure III. α₁-AR stimulation by phenylephrine attenuates the inhibitory effect of Rem1 on VLCC surface-membrane expression in concentration-dependent manor.

A. HEK293T cells were co-transfected with GFP-tagged Cav1.2 and α₁-AR. Cells were also transfected with either β₂α⁻ and/or α₂δ⁻ subunits. 24-hr after transfection, cells GFP-tagged Cav1.2 plasma membrane localization was observed by confocal microscope. Some cells were observed after 2-hr stimulation by 0.1-100 μmol/L phenylephrine (Gray bars). No-Rem1 transfected cells were also shows as control (Black bar). Membrane / cytosol (M / C) fluorescence ration was used for evaluation (see Material and Methods). > M/C ratio stands for no obvious plasma membrane localization (scattered line). *p<0.05, compared to the cells transfected with Rem1 before Phe stimulation (red bar).

B. Concentration-response curves for phenylephrine effect on VLCC surface-membrane expression in the presence of Rem1. The relationship between the normalized M /C ratio and the concentration of Phe was fitted with logistic equation.
Online Figure IV. Persistent $\alpha_1$-AR stimulation by phenylephrine attenuates the inhibitory effect of Rem1 on VLCC surface-membrane expression

**A.** Acute $\alpha_1$-AR stimulation (> 15 min) by phenylephrine did not attenuate the inhibitory effect of Rem1 on VLCC surface-membrane expression. HEK293T cells were co-transfected with VLCC subunits, $\alpha_1$-AR and Rem1. 24-hr after transfection, cells GFP-tagged Cav1.2 plasma membrane localization was observed by confocal microscope during 10 $\mu$mol/L phenylephrine stimulation. The representative pictures are time-dependently obtained from the same cell. Six experiments were done. **B.** Persistent $\alpha_1$-AR stimulation (> 1 hr) by phenylephrine attenuated the inhibitory effect of Rem1 on VLCC surface-membrane expression. The representative pictures from 6, 22 and 15 cells at 30min, 1hr and 4 hr after 10 $\mu$mol/L phenylephrine stimulation, respectively are shown. **C.** Summary data. *p<0.05, compared to control (0 min). #p<0.05, compared to 2-hr stimulation. N.S., no significance.
Online Figure V. $\alpha_1$-AR stimulation regulates the expression level of VLCCs at the plasma membrane through PKD1 activation.

A. $\text{Ca}^{2+}$ channel subunits and $\alpha^{1A}$-AR were co-transfected with (lane named Rem1, red bar) or without Rem1 (control, black bar) in HEK293T cells. Rem1-transfected cells were also stimulated with 10 $\mu$M phenylephrine (Phe) for 2 hours (Rem1+Phe, blue bar). The surface membrane proteins were collected by biotinylation method (see Material & Methods) and were blotted with anti-$\alpha_{1c}$-subunit antibody. Blotting of whole cell lysates and surface membrane proteins from non-transfected cells are also shown (no trans). B. VLCC subunits and Rem1 were co-transfected with (Rem1+PKD-SE, gray bar) or without PKD-SE (Rem1, red bar) in HEK293T cells. The cells transfected only $\text{Ca}^{2+}$ channel subunits were used as control (black bar). *$p<0.05$. N.S., no significance.
Online Figure VI. Acute $\alpha_1$-AR stimulation by phenylephrine did not show $I_{\text{Ca}}$ activation in HEK293T cells

VLCC subunits and $\alpha_{1A}$-AR were co-transfected with (B) or without (A, control) WT-Rem1 in HEK293T cells. 24-hr after transfection, whole-cell patch clamp was performed and $I_{\text{Ca}}$ was recorded during 10 $\mu$mol/L phenylephrine (Phe) stimulation for 5 min. Representative $I_{\text{Ca}}$ traces before (black open circle) and after Phe stimulation (red open circle) are showing in left panel. Holding potential was set at -80 mV and $I_{\text{Ca}}$ was observed by 500-msec depolalization pulse to +10 mV every 10sec. Vertical scale in A and B, 2pA/PF. Horizontal scale in A and B, 200ms. Average time course are shown in right panel. The averaged time course data of 4 experiments in each group are shown in right.
Online Figure VII. Acute stimulation by Cav1 agonist did not rescue Rem1-inhibited $I_{\text{Ca}}$ in HEK293T cells

A. Effect of Bay K 8644 on $I_{\text{Ca}}$ in the presence of absence of Rem1. VLCC subunits and $\alpha_{1A}$-AR were co-transfected with (bars at middle, n=5) or without (bars at left, n=5) WT-Rem1 in HEK293T cells. 24-hr after transfection, Rem-1 transfected cells were stimulated by 10 $\mu$mol/L Phe for 2 hr (bars at right, n=4). All groups were then stimulated by Cav1 agonist, 1 $\mu$mol/L Bay K 8644 for 10min and whole-cell patch clamp was performed and $I_{\text{Ca}}$ was recorded in the presence of Bay K 8644 (red bars). The currents evoked by 400-msec depolarization pulse to +10 mV were compared to those observed without Bay K 8644 treatment (black bars) (see also Figure 1D) in each group. B. Averaged Bay K 8644-induced fold-increase in $I_{\text{Ca}}$. There was no significant changes in the fold increases between 3 groups (control vs Rem1, $p=0.68$; Rem1 vs Rem1+Phe, $p=0.95$; control vs Rem1+Phe, $p=0.77$).
Online Figure VIII. \( \beta \)-AR stimulation did not attenuate the inhibitory effect of Rem1 on Ca\(^{2+} \) channel expression at the plasma membrane.

**A.** Sub-cellular localization of Ca\(^{2+} \) channels. Ca\(^{2+} \) channel subunits and \( \beta_1 \)-AR were co-transfected with (middle) or without Rem1 (control) in HEK293T cells. Rem1-transfected cells were also stimulated with either 100 nM isoproterenol (Iso) or adenylyl cyclase activator, 1 \( \mu \)mol/L forskorin (Forsk) for 2 hours (right). The profiles of fluorescence intensities from GFP-tagged \( \alpha_{1c} \)-subunits are shown below with the cross-section indicated in the pictures above by the red dot lines. **B.** Summary of the M/C ratios. Iso and Forsk stimulation did not increase Ca\(^{2+} \) channel expression at the plasma membrane in Rem1-transfected cells. The number of the cells used for analysis is shown in parentheses. *\( p<0.05 \), compared with control. N.S., no significance. **C.** \( \beta_1 \)-adrenoceptor stimulation did not show PKD1 activation and Rem1 phosphorylation on Serine 18. HEK 293T cells were transfected with Myc-WT-Rem1. After 24 h, cells were stimulated with PKA activator, 8-Br-cAMP (8-Bromo) or PKC activator, PMA for 30 min. PKD1 or Rem1 activation in cell lysates were determined by using phospho-PKD (Ser744/748) antibody (left) or phospho-Rem1 (Ser18) antibody (right) respectively. Total expression level of PKD1 or Rem1 was also shown. PKC activator activates PKD1 and induces Rem1 phosphorylation, but PKA activator did not.
Online Figure IX. $\alpha_{1B}$-AR and $\beta_1$-AR (but not $\alpha_{1A}$-AR) show agonists-mediated receptor internalizations.

GFP-tagged adrenergic receptors were transfected in HEK293T cells. 48-hr after transfection, cell was stimulated by either 10 $\mu$mol/L Phe (for $\alpha_1$-ARs) or 100 nmol/L isoproterenol (for $\beta_1$-AR) and localization of GFP-tagged receptors were time-dependently observed for 2hr at room temperature. Agonist-induced receptor internalizations were shown in allows.
Online Figure X. Rem1 phosphorylation site on serine 18 (in mouse) is conserved across eukaryotic species.
Alignment of Rem1 around Ser18 (in mouse) from 5 different species. The peptide sequences indicate that Rem1 has the potential PKD1 consensus motif and Rem1 phosphorylation site on serine 18 (in mouse) is conserved across eukaryotic species.

Online Figure XI. Generation of anti-phospho-Rem1 (Ser18) and its specificity in vivo.
Rabbit polyclonal antibody that can specifically recognize phosphorylated Rem1 at Ser 18 was commercially generated against a synthetic phosphopeptide C-Ahx-TLRRRA(pS)TPLPLS-amide corresponding to mouse Rem1 residues 12-24 (GenBank Accession Number NP_033073.1) (21st Century Biochemicals, Inc., Marlboro MA). Specificity of this custom-made antibody was tested using the whole cell lysates from the HEK293T cells transfected with WT- or mutant-Rem1 (S18A, S290A or S18A/S290A). This antibody recognized basal phosphorylation level of Rem1 at Ser18 and the phosphorylation levels increased in the cells co-transfected with PKD-SE.
Online Figure XII. PKD1 interacts with Rem1 and phosphorylates Rem1 at serine 18 in situ.

Hela cells were transfected with expression plasmids encoding Myc-tagged Rem1 with HA-tagged PKD1-WT or SE. Cells were lysed, and Myc-Rem1 was immunoprecipitated with anti-Myc Antibody. Immunoprecipitated proteins were subjected to SDS-PAGE followed by immunoblotting with anti-HA and with anti-Myc antibody. On the contrary, PKD1 were immunoprecipitated with anti-HA Antibody. Immunoprecipitated proteins were subjected to SDS-PAGE followed by immunoblotting with anti-Myc and with anti-HA antibody.
Online Figure XIII. Time-dependent effect of Phe on PKD activation and phosphorylation of Rem1 at Ser18.
HEK293T cells were co-transfected with $\alpha_1$-AR and Rem1 and were stimulated with 10 $\mu$mol/L Phe for 0.5-5 min. *$p<0.05$, compared to control (0 min).

Online Figure XIV. Concentration-dependent effect of Phe on PKD activation and phosphorylation of Rem1 at Ser18.
HEK293T cells were co-transfected with $\alpha_1$-AR and Rem1 and were stimulated with 0.1-10 $\mu$mol/L Phe for 2hr. *$p<0.05$, compared to control (0 min).
Online Figure XV. Phosphorylation of Rem1 at Ser18 by phenylephrine is mediated through α₁-AR stimulation.

HEK293T cells were transfected with α₁A-AR and Myc-Rem1. Cells were stimulated with phenylephrine (10 μM) for 30 min with or without selective α₁-AR antagonist, prazosin (1 μM). Lysates were subjected to SDS-PAGE followed by immunoblotting with anti-phospho-Rem1 (S18) antibody or anti-Myc antibody. *p<0.05. N.S., no significance.
Online Figure XVI. Expression of kinase-negative PKD1 blocks phenylephrine-induced VLCC activation through attenuating the Rem1 phosphorylation at Ser18.

A. α1A-AR and Myc-Rem1 were co-transfected with or without GFP-tagged kinase-dead PKD1 (PKD-DN) in HEK293T cell. Cells were stimulated with phenylephrine (10 µM) for 30 min. Lysates were subjected to SDS-PAGE followed by immunoblotting with anti-phospho-Rem1 (S18) antibody or anti-Myc antibody. Expression of PKD-KN attenuated α1A-AR-mediated Rem1 phosphorylation. *p<0.05. N.S., no significance.

B. Effect of α1-AR stimulation on current-voltage relationship of I_{Ca} in PKD-DN transfected cells. Ca^{2+} channel subunits, α1A-AR and Rem1 were co-transfected with GFP-tagged PKD-DN in HEK293T cells (open squares). Two-hour stimulation by phenylephrine did not rescue I_{Ca} activity in the cells co-transfected with PKD-DN (open triangles) (compare with blue circles in Figure 1D). PKD-DN itself did not change the basal I_{Ca} activity (compare open and closed circles). The number of the cells recorded is shown in parentheses.
Online Figure XVII. Phosphorylation of Rem1 by PKD1 regulates 14-3-3 binding.
Hela cells were transfected with expression plasmids encoding Myc-tagged Rem1-WT, S18A, S290A, S18A/S290A, without or with HA-tagged PKD-SE. Lysates were immunoprecipitated with anti-Myc Antibody. Immunoprecipitated proteins were subjected to SDS-PAGE followed by immunoblotting with anti-14-3-3β antibody to monitor 14-3-3 binding to Rem1. Equal loading was verified by anti-Myc antibody or anti-14-3-3β antibody from whole cell lysates. Data shows that phosphorylation of Rem1 at Ser18 by PKD1 increases the 14-3-3β binding to Rem1.
Online Figure XVIII. Phosphorylation of Rem1 by α₁-AR-PKD1 signaling regulates Rem1 localization from cytosol to nuclear.

A. Sub-cellular localization of GFP-tagged Rem1. HEK293T cells were transfected with expression plasmids encoding GFP-tagged Rem1-WT without or with HA-tagged PKD-SE. The profiles of fluorescence intensities from GFP-tagged Rem1 are shown below with the cross-section indicated in the pictures above by the red dot lines across nuclear area (left panels). Summary of the nuclear/cytosol (N/C) ratios of immunofluorescence intensity (right panel). PKD-SE significantly increased Rem1 localization at the nucleus. N/C ratio was calculated by using following formula: N/C ratio= (averaged nuclear florescence-averaged back ground florescence) / (averaged cytosol florescence-averaged back ground florescence). The number of the cells used for analysis is shown in parentheses. *p<0.05, compared with the cells transfected Rem1 alone (Black Bar).

B. Sub-cellular localization of GFP-tagged Rem1 and DsRed-ER maker. HEK293T cells were transfected with expression plasmids encoding GFP-tagged Rem1-WT, α₁A-AR and DsRed-ER maker (see Material and Methods). Cells were observed before (control) and after 2-hr stimulation by 10 µmol/L Phe. Before stimulation, GFP-tagged Rem was localized at cytosol and ER. After stimulation, cytosolyc localization of Rem1 was dramatically decreased and Rem1 was translocated to nucleus.
Online Figure XIX. α₁-AR stimulation activates PKD1 and induces Rem1 phosphorylation at Ser18 in neonatal cardiomyocytes.

A. Endogenous PKD1 was activated by α₁-AR stimulation in rat neonatal cardiomyocytes. Cells were incubated with phenylephrine (Phe, 10 µM) for various times as indicated and whole cell lysates were prepared. PKD1 activation and total amount of PKD1 were determined by western blot analysis using phospho-PKD (Ser744/748) antibody and anti-PKD1 antibody, respectively.

B. Rem1 was phosphorylated by α₁-AR-PKC-PKD1 signaling in rat neonatal cardiomyocytes. Neonatal cardiomyocytes were infected with adenovirus encoding control LacZ or Rem1 at 100 m.o.i. for 24 hours and then exposed to PMA (0.1 µmol/L) or Phe (10 µmol/L) for 30 min. Adenovirus encoding PKD-SE was also co-infected with control LacZ or Rem1 adenovirus into the cells. PKD1 or Rem1 activation in cell lysates was determined by western blot analysis using phospho-PKD (Ser744/748) or phospho-Rem1 (Ser18) antibody.
PKD-SE or Rem1 expression levels were also determined by western blot using anti-PKD1 antibody or anti-Rem1 antibody. Phosphorylation bands of Rem1 at Ser18 were observed when Rem1 was over-expressed, but phosphorylation levels of endogenous Rem1 were not detectable in whole cell lysates. C. Endogenous Rem1 was phosphorylated by $\alpha_1$-AR stimulation and PKD1 activation in rat neonatal cardiomyocytes. Cells were infected with PKD-SE or were stimulated with Phe. Cells were lysed, and immunoprecipitation was performed using anti-Rem1 Antibody. Immunoprecipitated proteins were subjected to SDS-PAGE, followed by immunoblotting with anti-phospho-Rem1 (Ser18) antibody. Whole cell lysates obtained after infection with Rem1 and PKD-SE was used as positive control of this blotting.
a. Summary of the membrane/cytosolic ratios of immunofluorescence intensity with or without stimulation of Phe (10 μmol/L, 2 hr) in native neonatal cardiomyocytes. Cells were fixed with or without stimulation of Phe and stained with anti-α1C-subunits antibody and sub-cellular localization of Ca\textsuperscript{2+} channels were observed. In this experiment, the cells were not infected with GFP-adenovirus. The representative cells and the profiles of immunofluorescence intensities are shown in Fig.4A. **p<0.01, compared with control.

b. Sub-cellular localization of Ca\textsuperscript{2+} channels (red) with (right) or without (left) α\textsubscript{1}-AR stimulation (10 μM Phe for 2 hours) in native neonatal cardiomyocytes. The cells were infected with GFP-adenovirus for comparing with the experiment results in Fig. 5B and C.
Online Figure XXI. $\alpha_1$-AR stimulation modulates subcellular localization of VLCC through Rem1 phosphorylation at Ser18 in neonatal cardiomyocytes.
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A. Representative confocal images of Cav1.2 localization in Rem1-overexpressed neonatal cardiac myocyte before (control) and after 2-hour treatment of 10 µmol/L Phe. Myc-tagged WT-Rem1 was overexpressed by the infection of adenoviral Rem1. Cav1.2 was strongly co-localized with Rem1 at cytoplasmic area (shown with white arrows). After Phe stimulation, Cav1.2 redistributed to the plasma membrane and cytosol and the degree of colocalization with Rem1 decreased. B. Representative Cav1.2 localization in Rem1 mutant (S18A)-overexpressed neonatal cardiomyocytes before (control) and after 2-hr treatment of Phe. Cav1.2 was strongly co-localized with Rem1S18A at cytoplasmic area (white arrows). However, after Phe stimulation, Cav1.2 did not change the subcellular localization and the colocalization of Rem1 and Cav1.2 did not alter. C. Summary data of quantitative colocalization analysis. Quantitative colocalization analysis was performed using Image J software (NIH). Colocalization was estimated using Pearson’s correlation coefficient (left) and Overlap coefficient according to Manders (right) (see Material and Methods). They range between 1 and -1 or 1 and 0, respectively with value of 1 being high co-localization, while low value being low co-localization. After Phe stimulation, co-localization of Rem1 and Cav1.2 significantly decreased but that of Rem1S18A and Cav1.2 did not change.
Online Figure XXII. Ca$^{2+}$ channel T-tubules localization does not change after 24-hr Rem1 infection in adult cardiomyocytes.

Subcellular localization of VLCC in adult ventricular myocytes 24-hr infection of LacZ (left) or Rem1 (right). The averaged profiles of immunofluorescence intensities from Cav1.2 staining (green) and plasma membrane marker WGA (red) from inset windows are also shown at the bottom.
Online Figure XXIII. \( \alpha_1 \)-AR stimulation induces \( \text{Ca}^{2+} \) channel T-tubules localization and
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increases $I_{Ca}$ in adult cardiomyocytes.

A. Subcellular localization of VLCC in the absence (left) and presence (right) of $\alpha_1$-AR stimulation (10 $\mu$M Phe for 2 hr) in adult ventricular myocytes infected with LacZ. The averaged profiles of immunofluorescence intensities from CaV1.2 staining (green) and plasma membrane marker WGA (red) from inset windows are also shown at the bottom. Scale bars in pictures, 2$\mu$m. B. Summary of power spectrum retrieved from CaV1.2 staining images (defined peak indicates the regular organization of the channel localization) in the absence (blue) and presence (red) of $\alpha_1$-AR stimulation infected with LacZ. FFT, Fast Fourier Transform. C. Effect of LacZ expression and Phe stimulation on current-voltage relationship of $I_{Ca}$. 
Online Figure XXIV. $\alpha_1$-AR stimulation increases $I_{Ca}$ through PKD activation in freshly-isolated adult cardiomyocytes.

A. Two-hr treatment of Phe increases $I_{CaL}$ in freshly isolated adult ventricular myocytes. Representative $I_{Ca}$ traces obtained from freshly isolated adult ventricular myocytes before (control, left) and after 2-hr treatment of 10 $\mu$mol/L Phe (right). Holding potential was set at -40 mV and 200-ms depolarization step pulse (from -80 mV to +60 mV by 10-mV increments) was applied to obtained current traces. B. Summarized current-voltage relationship before (control, black square) and after treatment of Phe (red circle).
Online Figure XXV. α₁-AR stimulation induces Rem1 phosphorylation at Ser18 in adult cardiomyocytes.

A. Adult ventricular myocytes were infected with adenovirus encoding control LacZ or Rem1 at 100 m.o.i. Whole cell lysates were made after 24-hr or 40-hr infection. Whole cell lysates from freshly isolated cells are also shown as control (0hr). Rem1 expression levels were determined by western blot using anti-Rem1 antibody. B. α₁-AR stimulation induces Rem1 phosphorylation at Ser18 in adult cardiomyocytes. Forty hour after infection with adenovirus encoding control LacZ or Rem1, cells were exposed to Phe (10 µmol/L) for 30 min. Rem1 phosphorylation in cell lysates was determined by western blot analysis using phospho-Rem1 (Ser18) antibody. Rem1 and CaV1.2 expression levels were also determined by western blot using anti-Rem1 and anti-CaV1.2 antibody. Phosphorylation bands of Rem1 at Ser18 were observed when Rem1 was over-expressed, but phosphorylation levels of endogenous Rem1 were not detectable in whole cell lysates.
Online Figure XXVI. α₁-AR stimulation enhances VLCC membrane expression through PKD1-dependent phosphorylation of Rem1 at Ser18 in cardiomyocytes.

A. Subcellular localization of VLCC in the absence (left) and presence (right) of α₁-AR stimulation (10 µmol/L Phe for 2 hours) in adult ventricular myocytes infected with Myc-tagged WT-Rem1 (Rem1). The profiles of immunofluorescence intensities from Ca_{V1.2} staining are also shown.

B. Subcellular localization of VLCC in the absence (left) and presence (right) of α₁-AR stimulation in adult ventricular myocytes infected with Rem1(S18A) (Rem1SA). The profiles of immunofluorescence intensity from Ca_{V1.2} staining are also shown.

C. Subcellular localization of VLCC in the absence (left) and presence (right) of α₁-AR stimulation (10 µmol/L Phe for 2 hr) in adult ventricular myocytes infected with GFP-tagged kinase-negative PKD1 (PKD-KN-GFP). The profiles of immunofluorescence intensities from Ca_{V1.2} staining in the region indicated are also shown.

D. Summary of FFT power spectrum retrieved from cell images in the absence (black) and presence (red) of α₁-AR stimulation infected with GFP.

E. Summary of FFT power spectrum retrieved from cell images in the absence (blue) and presence (red) of α₁-AR stimulation infected with PKD-KN. The data from GFP-infected cells is also shown as control (black).
Online Figure XXVII. Analysis methods for the quantification of sub-cellular localization of VLCCs

A. Plasma membrane localization of $\alpha_{1c}$-subunit was quantified by line scan intensity measurements through each cell beginning in the central cytoplasm (avoiding the nucleus) and ending at the cell periphery using Image J software (National Institutes of Health). Peak fluorescence intensity at the cell periphery in each scan was measured as “peak membrane fluorescence”. Membrane thickness was measured and was used for determining the background and cytosol region. The background and cytosol region were set at double length of averaged membrane thickness. See detail at supplementary Material and Methods.

B. The formula for calculate membrane/cytosol (M/C) ratio.

$$\text{membrane/cytosol (M/C) ratio} = \frac{\text{(peak membrane fluorescence - average background fluorescence)}}{\text{(average cytosol fluorescence - average background fluorescence)}}$$
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