Interaction of α1-Adrenoceptor Subtypes With Different G Proteins Induces Opposite Effects on Cardiac L-type Ca2+ Channel

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Abstract—We examined the effect of α1-adrenoceptor subtype-specific stimulation on L-type Ca2+ current (Ic₅ₒ) and elucidated the subtype-specific intracellular mechanisms for the regulation of L-type Ca2+ channels in isolated rat ventricular myocytes. We confirmed the protein expression of α₁ₐ- and α₁₇-adrenoceptor subtypes at the transverse tubules (T-tubules) and found that simultaneous stimulation of these 2 receptor subtypes by nonsubtype selective agonist, phenylephrine, showed 2 opposite effects on Ic₅ₒ (transient decrease followed by sustained increase). However, selective α₁ₐ-adrenoceptor stimulation (10 μmol/L A61603) only potentiated Ic₅ₒ, and selective α₁₇-adrenoceptor stimulation (10 μmol/L phenylephrine with 2 μ mol/L WB4101) only decreased Ic₅ₒ. The positive effect by α₁ₐ-adrenoceptor stimulation was blocked by the inhibition of phospholipase C (PLC), protein kinase C (PKC), or Ca²⁺/calmodulin-dependent protein kinase II (CaMKII). The negative effect by α₁₇-adrenoceptor stimulation disappeared after the treatment of pertussis toxin or by the prepulse depolarization, but was not attributable to the inhibition of cAMP-dependent pathway. The translocation of PKCα and ε to the T-tubules was observed only after α₁ₐ-adrenoceptor stimulation, but not after α₁₇-adrenoceptor stimulation. Immunoprecipitation analysis revealed that α₁ₐ-adrenoceptor was associated with Gq₁₁, but α₁₇-adrenoceptor interacted with one of the pertussis toxin-sensitive G proteins, Go. These findings demonstrated that the interactions of α₁-adrenoceptor subtypes with different G proteins elicit the formation of separate signaling cascades, which produce the opposite effects on Ic₅ₒ. The coupling of α₁ₐ-adrenoceptor with Gq₁₁-PLC-PKC-CaMKII pathway potentiates Ic₅ₒ. In contrast, α₁₇-adrenoceptor interacts with Go₅, of which the βγ-complex might directly inhibit the channel activity at T-tubules. (Circ Res. 2008;102:1378-1388.)

Key Words: α₁-adrenoceptor ▪ L-type Ca²⁺ channel ▪ G protein ▪ PKC

The α₁-adrenoceptor (AR) stimulation has an important role for the regulation of mammalian cardiac muscle contraction.¹⁻⁴ We have previously shown that α₁-AR stimulation modulates the function of voltage-gated L-type Ca²⁺ channels (VLCC) which is one of the important regulatory factors in cardiac excitation-contraction coupling.⁵ The effects of α₁-AR stimulation on cardiac Ca²⁺ current through VLCC (Ic₅ₒ) can be classified into 2 opposite effects (negative and positive effects): the positive effect is dependent on protein kinase C (PKC) and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) activity, but the negative effect is not.⁵ Although we have proposed this novel model for understanding the molecular mechanisms underlying the modulation of VLCC by α₁-AR stimulation, 2 important questions remain to be solved: (1) What is the molecular mechanism which simultaneously induces two opposite effects during α₁-AR stimulation?; (2) What are the molecular components for evoking the negative effect on Ic₅ₒ by α₁-AR stimulation? We postulated that these 2 opposite effects simultaneously occur via (1) different α₁-AR subtypes, α₁ₐ and α₁₇, which are the dominant receptor subtypes in mammalian heart¹⁻⁴ and (2) subtype-specific intracellular signal transduction pathways. The aims of this study are to characterize the effects of α₁-AR subtype-selective stimulation on Ic₅ₒ and to clarify the α₁-AR subtype-specific signaling pathway for the regulation of Ic₅ₒ. Here, we show the direct evidence that cardiac α₁-AR signaling diverges at the level of the α₁-AR subtype and G protein, which produce the opposite effects on Ic₅ₒ in rat ventricular myocytes. Alpha₁ₐ-AR coupled with Gq₁₁ and activated phospholipase C (PLC)-PKC-CaMKII pathway, which evoked the potentiation of Ic₅ₒ. In contrast, α₁₇-AR interacted with Go₅, of which the βγ-complex could directly inhibit Ic₅ₒ. These results represent the whole picture of intracellular mechanism for the unique regulation of VLCC by cardiac α₁-AR signaling and also provide the significant insight into the
Figure 1. Detection and cellular localization of $\alpha_1$-AR subtypes in rat ventricle. A, Detection of $\alpha_1$-AR subtypes in rat ventricle by Western immunoblot (IB) using specific antibodies against $\alpha_1A$- (left), $\alpha_1B$- (middle) or $\alpha_1D$-AR (right). Each well contained 50-μg membrane protein from rat ventricular myocytes (heart), urinary bladder (bladder), brain, or liver. B, Confocal images of isolated ventricular myocytes labeled with $\alpha_1$-AR subtype-specific antibody ($\alpha_1A$- or $\alpha_1B$-AR) (red, left) and the plasma membrane marker Wheat Germ Agglutinin-FITC (WGA) (green, middle). The overlay images are also shown (right). Bars=10 μm. C, Immunoelectron microscopic images of ventricular tissue labeled with 15-nm gold-$\alpha_1A$-AR (left) or $\alpha_1B$-AR (right). A high intensity of gold labeling was observed directly under T-tubule membranes (indicated by arrows). Mt indicates mitochondrion; Z, Z-line; Bar=500 nm.
regulation of cardiac excitation-contraction coupling by \(\alpha_1\)-AR subtype-specific signaling.

Materials and Methods

For details, please see the Data Supplement (available online at http://circres.ahajournals.org). Single ventricular myocytes and papillary muscles were prepared from adult male Wistar rats (300 to 400 g; Sankyo Labo Service, Tokyo, Japan). The measurement of \(I_c\), using a perforated patch clamp. Western immunoblot, immunoprecipitation, cAMP determination using an enzyme immunoassay, and immunofluorescence microscopy were performed on freshly isolated ventricular myocytes. Papillary muscles were used for immunoelectron microscopy. All results are shown as mean ± SD. Bars in the graphs indicate SD. Paired data were evaluated by Student \(t\) test. For multiple comparisons, 1-way or 1-way repeated ANOVA followed by Bonferroni post hoc test with the significance level set at \(P<0.05\).

Results

Detection and Cellular Localization of \(\alpha_1\)-AR Subtypes in Cardiomyocytes

The protein expression of \(\alpha_1\)-AR subtypes in isolated adult rat ventricular myocytes was confirmed by Western immunoblot with the commercially available antibodies against \(\alpha_1A\), \(\alpha_1B\), and \(\alpha_1D\)-AR (Figure 1A). In the membrane proteins from cardiomyocytes and urinary bladder, single bands were detected with the expected molecular size for glycosylated \(\alpha_1A\)-AR (68 kDa) using specific antibody against human \(\alpha_1A\)-AR (Figure 1A, left). However, in the parallel measurement with membrane proteins from rat brain, no positive band was observed. The specific antibody against human \(\alpha_1A\)-AR showed a major band with the expected molecular size for glycosylated \(\alpha_1B\)-AR (≈80 kDa) in rat cardiomyocytes, liver, and brain (Figure 1B, middle). The \(\alpha_1D\)-AR (60 kDa) was only found in rat brain; no significant bands were observed in cardiomyocytes and liver cells using the specific antibody against rat \(\alpha_1D\)-AR (Figure 1A, right). These results show that \(\alpha_1A\)- and \(\alpha_1B\)-AR (but not \(\alpha_1D\)-AR) were detectable at the protein level in our preparation of cardiomyocytes. Thus, we focused on the role of these 2 subtypes of \(\alpha_1\)-AR (\(\alpha_1A\) and \(\alpha_1B\)) in native cardiomyocytes in the following experiments.

We determined the cellular localization of \(\alpha_1A\)-AR and \(\alpha_1B\)-AR in cardiac cells using an immunofluorescence microscope (Figure 1B). In ventricular myocytes, \(\alpha_1A\)-AR was detectable at the plasmalemma and along the Z-lines, which coincides with the sarcosomal invaginations termed transverse tubules (T-tubules) where the majority of VLCC are located. On the other hand, \(\alpha_1B\)-AR was not detectable at the plasmalemma; rather it was localized at T-tubules and intercalated disks. The light microscopic images obtained from papillary muscle also showed a similar tendency of the localization of \(\alpha_1A\)-AR and \(\alpha_1B\)-AR as observed in the isolated cells (supplemental Figure 1).

To confirm the detailed subcellular localization of \(\alpha_1A\)-AR and \(\alpha_1B\)-AR, ultrathin cryosections of the left ventricular papillary muscles were incubated with these receptor subtype-specific antibodies (Figure 1C). The membranes of T-tubules were specifically labeled with the antibodies against \(\alpha_1A\)-AR and \(\alpha_1B\)-AR. These results suggest that 2 \(\alpha_1\)-AR subtypes (\(\alpha_1A\) and \(\alpha_1B\)) are detectable at the protein level in cardiac membrane, and they are preferentially localized at the T-tubules.

\(\alpha_1A\)-AR Stimulation Showed Only a Positive Effect on \(I_c\) Without a Negative Effect

\(\alpha_1A\)-AR stimulation by the nonsubtype selective agonist, 10 \(\mu\)mol/L phenylephrine (Phe), showed a biphasic change in \(I_c\) measured using the perforated patch clamp (a transient decrease followed by a sustained increase) in the presence of \(\beta\)-AR antagonist, 1 \(\mu\)mol/L bupranolol, which we used previously (Figure 2A). Similar results were obtained when we used another \(\beta\)-AR antagonist, 1 \(\mu\)mol/L propranolol, as shown in supplemental Figure II. Following experiments were all performed in the presence of 1 \(\mu\)mol/L bupranolol.

Next, we observed the effect of selective \(\alpha_1A\)-AR stimulation on \(I_c\) by using the selective \(\alpha_1A\)-AR agonist A61603. Fifteen-minute treatment with A61603 (0.1 \(\mu\)mol/L) evoked only potentiation of \(I_c\) (Figure 2B) without changing the current-voltage relationship (supplemental Figure III), and there was no negative effect in the initial period, which was observed in the presence of nonsubtype selective \(\alpha_1\)-AR stimulation by Phe (see Figure 2A). This positive effect after 15-minute treatment with A61603 was saturated at 1 \(\mu\)mol/L A61603 (0.1 \(\mu\)mol/L, 35.36±14.37%; n = 6; 1 \(\mu\)mol/L, 42.64±27.49%; n = 8; P = 1.00) and was blocked by the selective \(\alpha_1A\)-AR antagonist, 2 \(\mu\)mol/L WB4101 (n = 5, data not shown). All concentrations of A61603 (0.1 to 1 \(\mu\)mol/L) used showed only a positive without a negative effect (Figure 2C).

As we previously reported that the positive effect of \(\alpha_1\)-AR stimulation on \(I_c\) is evoked through a PKC- and CaMKII-dependent mechanism, next we investigated the involvement of PKC and CaMKII in the signaling pathways which evoke the potentiation of \(I_c\) during \(\alpha_1A\)-AR stimulation. In the presence of a PKC inhibitor chelerythrine, the positive effect of A61603 was not observed. CaMKII inhibition by KN-93 or autocamtide-2 inhibitory peptide (AIP; a membrane-permeable and a highly specific peptide type inhibitor of CaMKII) also abolished the potentiation of \(I_c\) by A61603 (Figure 2D). Moreover, in the presence of a PLC inhibitor, U73122, the positive effect of A61603 completely disappeared (Figure 2D). These results suggest that \(\alpha_1A\)-AR stimulation shows only a positive effect on \(I_c\), and this effect is mediated through the PLC-PKC-CaMKII pathway.

\(\alpha_1B\)-AR Stimulation Showed Only a Negative Effect on \(I_c\) Without a Positive Effect

We investigated the effect of \(\alpha_1B\)-AR stimulation on \(I_c\) by the application of a nonsubtype selective \(\alpha_1\)-AR agonist (Phe) with a selective \(\alpha_1A\)-AR antagonist (WB4101), because no selective \(\alpha_1B\)-AR agonist is available at present. Ten-minute exposure to 2 \(\mu\)mol/L WB4101 significantly decreased \(I_c\) without changing the shape of the current-voltage relationship (supplemental Figure III) and reached another steady state (Figure 2F, red diamonds). In the continuous presence of WB4101, 10 \(\mu\)mol/L Phe only decreased \(I_c\) (Figure 2E and 2F) without changing the shape of the current-voltage relationship (supplemental Figure III). In contrast, 1 \(\mu\)mol/L Phe (no negative effect on \(I_c\)) was observed at this concentra-
Figure 2. Alpha1A-AR stimulation shows only positive effect in \( I_{Ca} \), but \( \alpha_{1B}-\text{AR} \) stimulation shows only negative effect in \( I_{Ca} \). A, A representative record of the time-dependent change in \( I_{Ca} \) during the application of the nonsubtype selective \( \alpha_{1A}-\text{AR} \) agonist, 10 \( \mu \text{mol/L} \) Phe. B, A representative record of time-dependent change in \( I_{Ca} \) during application of the selective \( \alpha_{1A}-\text{AR} \) agonist, 0.1 \( \mu \text{mol/L} \) A61603. C, Concentration-dependent effect of A61603 on \( I_{Ca} \). Time course of \( I_{Ca} \) in the absence of A61603 is also shown (red diamonds). The amplitudes of currents at each period were normalized by the current before the application of A61603. The number of the cells tested is shown in parentheses. * \( P < 0.05 \), ** \( P < 0.01 \) compared to the normalized current in the absence of A61603 (red diamonds) at each time. 

D, Effect of 0.1 \( \mu \text{mol/L} \) A61603 on \( I_{Ca} \) in the presence of PLC inhibitor (1 \( \mu \text{mol/L} \) U73122), PKC inhibitor (10 \( \mu \text{mol/L} \) chelerythrine), or CaMKII inhibitors (0.5 \( \mu \text{mol/L} \) KN-93 or 10 \( \mu \text{mol/L} \) AIP). One \( \mu \text{mol/L} \) U73343 and 0.5 \( \mu \text{mol/L} \) KN-92 were also applied as the inactive analogues of U73122 and KN-93, respectively. Each inhibitor or inactive analogue was applied 10 minutes before the agonist application. Graphs show the ratios of \( I_{Ca} \) 15 minutes after application of A61603 to that before the application of A61603. The number of the cells tested is shown in parentheses. * \( P < 0.05 \), compared to the control (0.1 \( \mu \text{mol/L} \) A61603). † \( P < 0.05 \), compared to the current in the presence of inactive form analogues (KN-92 or U73343). 

E, A representative time course of \( I_{Ca} \) during application of 10 \( \mu \text{mol/L} \) Phe in the presence of selective \( \alpha_{1B}-\text{AR} \) antagonist, 2 \( \mu \text{mol/L} \) WB4101. 

F, Concentration-dependent effect of Phe in the presence of 2 \( \mu \text{mol/L} \) WB4101 on \( I_{Ca} \). WB4101 was applied 10 minutes before agonist application. The amplitudes of currents at each period were normalized by the current before the application of Phe. The number of the cells tested is shown in parentheses. * \( P < 0.05 \) compared to the normalized current in the absence of Phe (red diamonds) at each time. 

G, Concentration-dependent effect of the selective \( \alpha_{1B}-\text{AR} \) antagonist, L765,314 on a transient decrease in \( I_{Ca} \) 2 minutes after application of 10 \( \mu \text{mol/L} \) Phe. L765,314 was applied 10 minutes before agonist application. Graphs show the percent inhibition of \( I_{Ca} \) 2 minutes after application of 10 \( \mu \text{mol/L} \) Phe in the presence (10 to 100 nmol/L, \( n=4 \)) or in the absence of L765,314 (0 nmol/L, \( n=12 \)). * \( P < 0.05 \), compared to the control (0 nmol/L L765,314).
stimulation on I$_{Ca}$, in the presence of WB4101, showed no significant positive or negative effects (Figure 2F). Thus, $\alpha_{1b}$-AR stimulation produced only a negative effect without potentiation of $I_{Ca}$, which was opposite to the effect of $\alpha_{1a}$-AR stimulation.

To further confirm the negative effect by $\alpha_{1b}$-AR stimulation on $I_{Ca}$, we also investigated the effect of 10 $\mu$mol/L Phe in the presence of the selective $\alpha_{1b}$-AR antagonist, L-765,314. The negative effect of 10 $\mu$mol/L Phe on $I_{Ca}$ was significantly inhibited by the treatment of L-765,314 in whole cell lysates (W) are also shown (50 $\mu$g protein/well). Control (CTR) represents untreated cells. Total PKC$\alpha$, $\delta$, and $\varepsilon$ in whole cell lysates (W) are also shown (50 $\mu$g protein/well). D to F, Graphs show M/C ratios for the evaluation of PKC-isoform-specific translocation (n=7). The M/C ratio after each stimulation (Phe, $\alpha_{1a}$, $\alpha_{1b}$, or PMA) was normalized by the M/C ratio before stimulation (CTR). *P<0.05, compared to the control (CTR).

**PKC Was Activated After $\alpha_{1a}$-AR Stimulation, but Not After $\alpha_{1b}$-AR Stimulation**

We previously showed that the positive effect of $\alpha_{1a}$-AR stimulation on $I_{Ca}$, as dependent on PKC activity. Therefore, we examined the involvement of PKC in the signaling pathway after $\alpha_{1a}$- or $\alpha_{1b}$-AR stimulation. One of the hallmarks of PKC activation is the translocation of soluble enzymes to particle fractions, presumably near their protein substrates that include sarcolemmal proteins. We determined the subcellular localization of the activated PKC isozymes to elucidate their roles in the regulation of VLCC before and after selective $\alpha_{1a}$-AR-subtype stimulation by using an immunofluorescence microscope (Figure 4A to 4C). Significant translocation of PKC$\alpha$ was not observed after the treatment with Phe as shown in Western immunoblot (Figure 4A). Most of PKC$\delta$ was localized in the nucleus or at the nuclear membrane, but the remainder was diffusely distributed in the cytosol at rest (Figure 4B). After Phe treatment, a striated pattern also became visible, which was in accordance with the location of T-tubules (Figure 4B). PKC$\varepsilon$ was diffusely distributed in the cytosol before stimulation (Figure 4C). After Phe treatment, PKC$\varepsilon$ was accumulated at the T-tubules and intercalated disks (Figure 4C).

These results suggest that Ca$^{2+}$-independent PKCs were activated and the activated PKCs were redistributed to the membrane fraction, presumably to the T-tubules after $\alpha_{1a}$-AR stimulation.
stimulation. However, there was no obvious involvement of PKC in the α_{1B}-AR signaling pathway.

**Negative Change in I_{Ca} During α_{1}-AR Stimulation Was Mediated via the Pertussis Toxin–Sensitive G Protein Pathway**

We showed that the positive effect on I_{Ca} caused by α_{1A}-AR stimulation was dependent on PKC, but the negative effect of α_{1B}-AR stimulation was independent of PKC activation (Figures 2 and 3). Several reports demonstrated that α_{1}-AR couples not only with G_{q/11} which in turns leads to activation of PLC and PKC, but also with the pertussis toxin (PTX)-sensitive G proteins, and it shows diverse physiological effects in cardiomyocytes.\(^1\)\(^4\)\(^12\) Therefore, we hypothesized that α_{1B}-AR functionally couples with other G proteins, and we examined the involvement of PTX-sensitive G protein in the regulation of I_{Ca} by α_{1}-AR stimulation.

Inhibition of G_{α_{1B}}-protein by PTX in our preparations was confirmed by the ability of PTX to block the muscarinic inhibition of I_{Ca} in the presence of β-AR stimulant (supplemental Figure VI). Treatment of PTX significantly inhibited the negative effect by 10 μmol/L Phe at 2 minutes and then enhanced the positive effect at 15 minutes (Figure 5A). Moreover, we separately investigated the effects of α_{1A}-AR subtype-selective stimulation on I_{Ca} in PTX-treated cells. We confirmed that the negative effect by α_{1B}-AR stimulation was blocked by PTX (Figure 5C), but the magnitude of the positive effect by α_{1A}-AR stimulation did not alter after PTX treatment (Figure 5B). These results indicate that the negative
phase of $I_{Ca}$ during $\alpha_{1B}$-AR stimulation (Figure 2A) was produced through PTX-sensitive G protein (G$_i/o$) pathways. In adult rat cardiomyocytes at least 3 subtypes of PTX-sensitive G$_i/o$ (G$_i$-2, G$_i$-3, G$_o$) are expressed at the mRNA level and are detectable at the protein level. Therefore, the possibility that $\alpha_1$-AR subtypes directly couple with these PTX-sensitive G proteins was examined by coimmunoprecipitation of these G$_i/o$-subunits with anti-$\alpha_1A$- or anti-$\alpha_1B$-AR antibody. The immunoprecipitants were analyzed by Western immunoblot probing with the antibodies against G$_i/o$-subunits.

**Figure 5.** Negative effect on $I_{Ca}$ during $\alpha_1$-AR stimulation is mediated through $\alpha_1B$-AR and PTX-sensitive G protein pathway. A to C, Effect of 10 $\mu$mol/L Phe (A), 1 $\mu$mol/L A61603 (B), or 10 $\mu$mol/L Phe with 2 $\mu$mol/L WB4101 (C) on $I_{Ca}$ in PTX-treated cells (red circles) and in non-treated cells (black circles). The amplitudes of currents at each period were normalized by the current before the application of Phe. *P<0.05, compared to the normalized current in the PTX-nontreated cells (black circles) at each time. The number of the cells tested is shown in parentheses. D, Coimmunoprecipitation of $\alpha_1$-ARs with G$_i/o$-subunits. Membrane proteins were immunoprecipitated (IP) with specific $\alpha_1$-AR antibodies or control IgG (IgG). The immunoprecipitates were analyzed by Western immunoblot by probing with the antibodies against $\alpha_1$-ARs and G$_i/o$-subunits. Membrane lysates (ML; 12.5 $\mu$g/lane) are also shown as the positive control. E, Immunofluorescence images of ventricular myocytes costained with antibodies against anti-G$_o$ (red, upper panel) and $\alpha_1B$-AR (green, middle panel). The overlay image is also shown (lower panel). Bars, 10 $\mu$m.
of which specificities were checked by using the recombinant Gα-subunits (see supplemental Figure VII). The α1α-AR antibody coimmunoprecipitated Gαq/11, whereas the α1β-AR antibody coimmunoprecipitated Gαo (Figure 5D). Moreover, immunofluorescence images with the specific antibodies against ααααααα-AR and Gαo showed that Gαo was colocalized with α1β-AR at T-tubules (Figure 5E). Thus, these results indicate that the ααααααα-AR couples with Gαq/11-protein in a classical coupling mode, which activates the PLC-diacylglycerol-PKC pathway and that α1β-AR is linked to Gαo at the T-tubules and evokes the negative phase in I_{Ca}.

**Negative Effect of α1-AR Stimulation on I_{Ca} Is Mediated Through βγ-Complex of G Protein**

Our biochemical and electrophysiological results indicated that α1β-AR interacted with one of the PTX-sensitive G proteins, Gα. However, the functional roles of Gα-protein in native cardiomyocytes have not been clarified. We postulated here that α1β-AR-Gα interaction could inhibit the VLCC activity through (1) the decrease of basal cAMP level (eg, by the inhibition of adenylyl-cyclase activity as in the case of Gγ3), or (2) stimulation of protein phosphatase activity, followed by the reduction of basal phosphorylation level of the VLCC. However, we found that the basal cAMP level in our preparations did not significantly change during α1-AR stimulation as described previously14 (Figure 6A), and negative effect of I_{Ca} by Phe was clearly observed even in the presence of cAMP-dependent protein kinase (PKA) inhibitor (1 μmol/L H-89; Figure 6B). Thus, the inhibition of cAMP-PKA signaling is not involved in the mechanism for evoking negative phase of I_{Ca}. Moreover, we pretreated the cells with a protein phosphatase inhibitor, calyculin A in the presence of H-8915 and then investigated the effects of Phe in the continuous presence of calyculin A and H-89. Under this condition,15 we still observed the negative phase of I_{Ca}, suggesting that activation of phosphatases followed by the reduction of basal VLCC phosphorylation is not involved in the negative phase (supplemental Figure VIII).

Several reports stated that the βγ-complex of heterotrimeric Gα-protein directly interacts with N-type or L-type Ca^{2+} channels to inhibit their activity.16,17 Moreover, a depolarization pulse applied to the membrane before channel activation is known to counteract this inhibition.16 Therefore, we next observed the effect of a nonsubtype selective α1-AR agonist (10 μmol/L Phe) on I_{Ca} using this prepulse depolarization protocol (Figure 7A).

Recording with this prepulse depolarization, there was no significant transient decrease of I_{Ca} for up to 2 minutes after the application of 10 μmol/L Phe (Figure 7A and 7B). Fifteen minutes after the application of Phe, I_{Ca} was significantly increased (Figure 7A and 7B). Thus, the current inhibition at
the initial stage (≈2 minutes) induced by α1-AR stimulation was not attributable to the reduction of basal phosphorylation level of VLCC, but was possibly produced by the direct interaction of βγ-complex of Go with VLCC.

**Discussion**

In this study, we elucidated the differences between cardiac α1A- and α1B-AR signaling pathways and provide direct evidence indicating that different G proteins (and kinases) are involved in the respective subtype-specific signaling pathway and induce opposite changes in I_{Ca} in native cardiomyocytes (Figure 8). We showed that α1A- and α1B-AR were functionally expressed at T-tubules where VLCC is concentrated, but α1D-AR was not detected at protein level and was not functionally expressed in our preparations (supplemental Figure IV). Furthermore, we clearly separated the effect of α1A- or α1B-AR stimulation from that of nonsubtype selective stimulation on I_{Ca} by pharmacological procedure and clarified the detail of each signaling pathway by biochemical and morphological techniques. Alpha_{1A}-AR and alpha_{1B}-AR signaling pathways couple with different G proteins, G_{q/11} and G_{α}, respectively and produce different functional outcomes; α1A-AR stimulation activates G_{q/11}-PLC-diacylglycerol-PKC-CaMKII pathway and increases I_{Ca}. On the contrary, α1B-AR interacts with Go and inhibits the VLCC activity.

**Alpha_{1A}-AR-Gq-PKC Signaling Pathway Induces Potentiation of I_{Ca}**

In this study, we showed that α1A-AR was expressed at T-tubules and also demonstrated that α1A-AR stimulation did affect the VLCC activity which was confirmed by using α1A-AR selective agonist, A61603. Alpha_{1A}-AR pathway potentiated I_{Ca} in native cardiomyocytes, which is mediated through a PKC-dependent mechanism (Figure 8). PKC is a phospholipid-dependent Ser-Thr kinase, and most isoforms of the PKC are activated as a result of receptor-dependent activation of PLC and the hydrolysis of membrane phosphoinositides. Although all cloned subtypes of α_{1A}-AR can induce PLC activation and inositol phosphate formation, receptor subtype-specific activation or downregulation of PKC has been reported in cultured neonatal cardiomyocytes. In cardiac tissue, the isoforms of Ca_{2+}-dependent PKC (PKCα) and 2 Ca_{2+}-independent PKCs (novel PKCs; PKCδ and PKCε) are at least detectable at the protein level. In our preparations, we demonstrated that only α1A-AR stimulation induces the activation of novel PKCs and translocates them to the cell membrane structure called T-tubules,
and that α₁β₁-AR stimulation did not show any activation of PKC. This result is consistent with the previous report that α₁γ₉- or α₁γ₇-AR signaling pathway does not have any influences on PKC activity.¹⁹ We did not detect any significant activation of PKCα after α₁-AR stimulation, indicating that PKCα was not involved in α₁-AR signaling¹⁹ in our preparations. Moreover, we directly showed the interaction of α₁α₁-2AR and Gₛ₁₁ which activates PLC.¹⁸ The α₁α₁-AR-Gₛ₁₁-PLC-diacylglycerol pathway activated novel PKCs and translocated them to T-tubules where VLCC and CaMKII are prevalent.⁵²¹ The translocated PKC could activate CaMKII at T-tubules,⁵ and then the activated CaMKII could directly potentiate Iₛᵣ through the phosphorylation of α₁, and/or β subunits of the channel.²¹²² Thus, α₁α₁-AR signaling components preferentially localized at the T-tubules and efficiently regulated cardiac VLCC, as in the case of cardiac endothelin-receptor signaling.²³

**Alpha₁β₁-AR-G₉ Interaction Induces the Inhibition of Iₛᵣ**

We showed that α₁β₁-AR was expressed at T-tubules and also demonstrated that α₁β₁-AR stimulation did affect the VLCC activity confirmed by pharmacological procedures. Alpha₁β₁-AR stimulation inhibited Iₛᵣ, which is mediated through PKC-independent mechanisms (Figure 8). Our biochemical studies indicated that α₁β₁-AR stimulation shows less influence on the PKC activity than α₁α₁-AR stimulation. This result is compatible with the previous reports that α₁γ₉-AR subtype has less potency for the stimulation of phosphoinositide hydrolysis than α₁α₁-AR.²⁴²⁵ We found that α₁β₁-AR coupled with Gₛᵣ instead of Gₛ₁₁ and this pathway brought about negative modulation of Iₛᵣ, which was not attributable to the decline of basal phosphorylation level of VLCC by the inhibition of cAMP-PKA pathway or activation of protein phosphatases (Figure 6 and supplemental Figure VIII). Our result is consistent with the previous results obtained using a constitutively active mutant of α₁β₁-AR or the technique of the overexpression of wild-type α₁β₁-AR, which shows the possibility that only α₁β₁-AR (but not α₁α₁-AR) couples with the PTX-sensitive pathway.²⁶²⁷ Iₛᵣ inhibition through Gₛᵣ was reported in secretory cells²⁷ and cardiac cells from genetically engineered mice,²⁸ but the functional role of Gₛᵣ in native cardiomyocytes is still poorly understood. Ivanina et al showed that the direct binding of Gₛᵣ subunit of Gₛᵣ to VLCC inhibits the channel activity in heterologous expression systems.²⁹ They also reported that the basal intracellular Ca²⁺ level is essential for this inhibition, which is consistent with our previous data.⁵ We also showed that α₁β₁-AR and Gₛᵣ colocalize with VLCC at T-tubules, and we propose the working model that βγ-complex of Gₛᵣ protein could directly inhibit VLCC.

**Conclusion**

In conclusion, our results represent the evidence that the unique combinations of α₁-AR subtypes and specific G proteins form subtype-specific signal transduction pathways, which induce the opposite effects on VLCC in native cardiac cells. The coupling of specific α₁-AR subtypes with PTX-sensitive G protein could exhibit the negative feedback response to α₁-AR stimulation, and this mechanism would contribute to the protection of the heart from Ca²⁺ overload as in the relation between β₁- and β₁-AR. The approach of characterizing the receptor subtype-specific interacting G protein will provide new insight to elucidate the whole picture of the subtype-specific signaling pathway in native cardiomyocytes, and further could lead to an understanding of the functional roles of each α₁-AR subtype under physiological and pathophysiological condition.

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**Disclosures**

None.

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