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Abstract
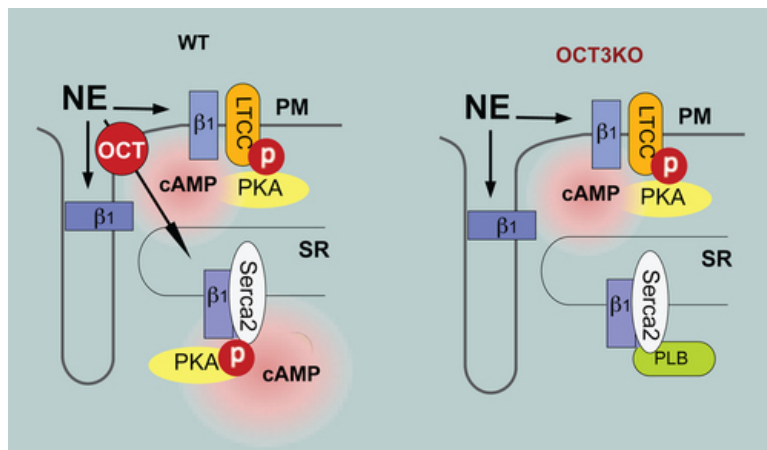
Rationale:

β_1ARs (β_1-adrenoceptors) exist at intracellular membranes and OCT3 (organic cation transporter 3) mediates norepinephrine entry into cardiomyocytes. However, the functional role of intracellular β_1AR in cardiac contractility remains to be elucidated.

Objective:

Test localization and function of intracellular β_1AR on cardiac contractility.

Methods and Results:

Membrane fractionation, super-resolution imaging, proximity ligation, coimmunoprecipitation, and single-molecule pull-down demonstrated a pool of β_1ARs in mouse hearts that were associated with sarco/endoplasmic reticulum Ca^{2+}-ATPase at the sarcoplasmic reticulum (SR). Local PKA (protein kinase A) activation was measured using a PKA biosensor targeted at either the plasma membrane (PM) or SR. Compared with wild-type, myocytes lacking OCT3 (OCT3-KO [OCT3 knockout]) responded identically to the membrane-permeant βAR agonist isoproterenol in PKA activation at both PM and SR. The same was true at the PM for membrane-impermeant norepinephrine, but the SR response to norepinephrine was suppressed in OCT3-KO myocytes. This differential effect was recapitulated in phosphorylation of the SR-pump regulator phospholamban. Similarly, OCT3-KO selectively suppressed calcium transients and contraction responses to norepinephrine but not isoproterenol. Furthermore, sotalol, a membrane-impermeant βAR-blocker, suppressed isoproterenol-induced PKA activation at the PM but permitted PKA activation at the SR, phospholamban phosphorylation, and contractility. Moreover, pretreatment with sotalol in OCT3-KO myocytes prevented norepinephrine-induced PKA activation at both PM and the SR and contractility.

Conclusions:

Functional β_1ARs exists at the SR and is critical for PKA-mediated phosphorylation of phospholamban and cardiac contractility upon catecholamine stimulation. Activation of these intracellular β_1ARs requires catecholamine transport via OCT3.

Introduction

Plasma membrane receptors are major mechanisms by which cells respond to extracellular stimuli, allowing cells to adapt to their surrounding environments and to modulate tissue/organ function in reaction to changes of neurohormones. Nonsteroid hormones signal mainly through receptors at the plasma membrane (PM) to regulate cellular function. This includes sympathetic regulation of cardiac function via ARs (adrenergic receptors) on the PM of cardiomyocytes. Recently, a complementary thread of studies has found that neurotransmitters, peptide hormones, and growth factors also act on intracellular membrane receptors.[1–4] Accumulating data indicate that intracellular GPCRs (G-protein coupled receptors) located at different organelles, for example, mitochondria,[5] endoplasmic reticulum membranes,[6] and Golgi,[7] can be activated insides the cells and induce location-specific effects. The development of these observations helps expanding the current understanding of hormonal regulation of cardiac function.

β_1ARs (β_1-adrenoceptors), a prototype GPCR, acts as a linchpin of sympathetic regulation in the heart and has been demonstrated to be localized and activated intracellularly in cardiomyocytes.[8] However, the role of intracellular β_1ARs in the regulation of cardiac excitation-contraction is still unknown. During the fight-or-flight response, activation of the sympathetic nerve and adrenal glands releases catecholamines (epinephrine, and norepinephrine) to activate βARs on myocytes and thus enhance heart rate, cardiac contraction and relaxation.[9,10] These effects involve βARs stimulation-dependent cAMP (cyclic adenosine monophosphate) and subsequent activation of PKA (protein kinase A) to enhance substrate phosphorylation. The PKA phosphorylation of different substrates occurs in different spatiotemporal nanodomains including LTCC (L-type Ca^{2+} channel) at the PM, PLB

(phospholamban) and RyR2 (ryanodine receptor type 2) on the sarcoplasmic reticulum (SR), and TnI (troponin I) and MYBP-C (myosin binding protein C) on the myofilaments.[11,12]

Both norepinephrine and epinephrine are vital neurohormones in physiological stress responses. However, epinephrine and norepinephrine have extremely hydrophilic octanol/water partition coefficients (Log$P$ =−1.37 and −1.24, respectively, https://pubchem.ncbi.nlm.nih.gov). After release from nerve termini, a small portion of catecholamine can enter myocytes, primarily mediated by corticosterone-sensitive OCT3 (organic cation transporter 3, also named EMT [extraneuronal monoamine transporter]).[2,13] OCT3-mediated norepinephrine uptake is necessary for activation of intracellular ARs in cardiomyocytes, including both nuclear α_1ARs and Golgi-localized β_1ARs.[2,8] Intriguingly, decreased norepinephrine uptake into myocardium and cardiac norepinephrine contents in patients with heart failure (HF) are associated with significantly blunted inotropy response.[14] In dilated cardiomyopathy patients, OCT2 expression is reduced and predicts the impairment of cardiac function.[15] However, the functional role of norepinephrine uptake facilitated by OCT3 in adrenergic stimulation of cardiac contractile is unclear.

Activation of β_1ARs induces spatial, temporal segregated cAMP-PKA signals in heart.[12,16] The application of Förster resonance energy transfer (FRET)–based biosensors have been instrumental in the investigation and dissection of compartmentalized cAMP/PKA signaling on the nanodomain scale.[12,17,18] By using genetically encoded biosensors, previous studies from our group and others found that compartmentalized cAMP/PKA signal generated in response to βAR stimulation differs at the PM and SR local domains in both amplitude and kinetics.[12,17] The mechanism underlying this heterogeneity is still unclear. We hypothesize that a population of β_1ARs is located near or at the SR and plays an important role in modulating calcium (Ca^{2+}) cycling and cardiac contractility. In this study, we tested the expression, activation, and functional effects of SR-localized β_1AR in cardiac contractility. Our results provide novel insights into the localization and regulation of intracellular β_1AR, therefore, offering potential and precise target for β_1AR-associated therapeutic strategies.

Methods

Data Availability
The data, analytic methods, and study materials are made available to other researchers for purposes of reproducing the results or replicating the procedure. Please see the Major Resources Table and Expanded Materials & Methods in the Data Supplement.

All experiments including animal feeding, treatment, and tissue collection were approved by the Institutional Animal Care and Use Committees (protocol: 20234 and 20957) of the University of California at Davis and follow National Institutes of Health guidelines. Male and female 2- to 4-month-old C57/BL6 and OCT3-KO, newborn β_1AR-KO, β_2AR-KO, and $\beta_1\beta_2$AR-KO mice were described previously.[17,19] Male Sprague Dawley outbred rats and New Zealand white rabbits were used in this study.

Results

Existence and Distribution of a Distinct Pool of β_1AR at the SR in Cardiomyocytes

To examine the existence of different subcellular pools of β_1ARs in adult mouse hearts, we fractionated heart tissues to separate the PM from intracellular membrane compartments. SERCA2 (sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase 2) and RyR2, which are SR resident proteins, were used as markers for the intracellular membrane fraction (non-PM), and InsR (insulin receptor) and lack of SERCA2/RyR2 were used to identify PM fraction. β_1ARs were clearly detected in both PM and intracellular membrane fractions (non-PM) in wild-type (WT) mouse hearts but not in β_1AR-KO mouse hearts (Figure 1A and Figure IA in the Data Supplement), indicating that endogenous β_1AR exists in at least 2 different pools in mouse hearts. In addition, quantitative radioligand-binding assay measured the functional β_1ARs that bind to [I^{125}] radio-labeled cyanopindolol in both PM and intracellular membrane fractions (Figure 1B), supporting the existence of an intracellular pool of functional β_1ARs in hearts. We also applied a fluorescence dye BODIPY-tagged βAR antagonist propranolol (BODIPY-tagged propranolol [FL-Prop]) to stain endogenous β_1ARs in mouse adult ventricle myocytes (AVMs). Confocal images of FL-Prop fluorescent staining in WT mouse AVMs showed a distribution of βARs on both surface membrane and intracellular space (Figure IB in the Data Supplement), which was abrogated by addition of 1 μmol/L and 10 μmol/L propranolol (Figure IB in the Data Supplement). Moreover, compared with WT and β_2AR-KO cardiomyocytes, the fluorescent dots and intensity of FL-Prop were greatly reduced in the β_1AR-KO and $\beta_1\beta_2$AR-KO cardiomyocytes (Figure IC and ID in the Data Supplement), indicating that FL-Prop mainly stains endogenous β_1AR.
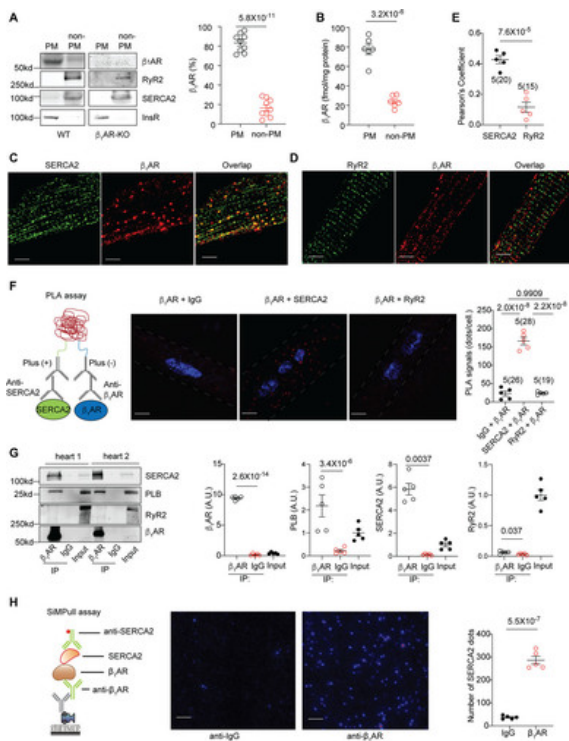
Figure 1. Intracellular β1AR (β$_1$-adrenoceptors) associates with SERCA2 (sarcoplasmic endoplasmic reticulum Ca2+-ATPase 2) but not RyR2 (ryanodine receptor type 2) in hearts and isolated mouse adult ventricular cardiomyocytes (AVMs).A, Wild-type (WT) and β$_1$AR-knockout (KO) mouse heart lysates were fractionated to assess cellular distribution of β$_1$AR. Representative blots and the percentages of β$_1$AR in the plasma membrane (PM) and intracellular membrane fractions (non-PM) over the total β$_1$AR in WT and β$_1$AR-KO mouse hearts. Data

are shown in mean±SEM. N=9 mice. *P* values were obtained by Student *t* test. **B**, β_1AR densities in the PM and non-PM fractions of WT mouse hearts were determined by quantitative radioligand-binding assay. Data are shown in mean±SEM. N=6 mice. *P* values were obtained by Student *t* test. **C** and **D**, Representative confocal images showing distribution of β_1AR, SERCA2, and RyR2 in isolated mouse AVMs. β_1AR was overexpressed in AVMs by infection with recombinant adenovirus. Scale bar=5 μm. **E**, The overlap between staining from confocal images was evaluated by Pearson correlation coefficient using ImageJ. Data are shown in mean±SEM; AVM (in the parenthesis) and mouse numbers are indicated in the figure. *P* value was obtained by *t* test. **F**, Schematic of in situ proximity ligation assay (PLA), exemplary fluorescence images (the whole-cell images are in Figure II in the Data Supplement) and quantification of PLA signals after labeling with antibodies against β_1AR and IgG, β_1AR and SERCA2, and β_1AR and RyR2 in AVMs, respectively. Positive PLA signal (red), DAPI (4′,6-diamidino-2-phenylindole) (blue). Scale bar=5 μm. Data are shown in mean±SEM; AVM (in the parenthesis) and mouse numbers are indicated. *P* values were obtained by 1-way ANOVA followed by Tukey multiple comparison test. **G**, Representative images and quantitative assessment of PLB (phospholamban), SERCA2, and RyR2 coimmunoprecipitated with β_1AR in WT mouse hearts. Arbitrary unit (A.U) is defined as the ratio of intensity of proteins over inputs. Data are shown in mean±SEM (N=5); *P* values were obtained by Student *t* test; **H**, Schematic of supersensitive single-molecule pull-down (SiMPull) assay and representative images of SiMPull assay after endogenous β_1AR, SERCA2 complex were pulled down with anti-β_1AR or control IgG antibodies. The images were quantified using MATLAB. Scale bar=5 μm. Data are shown in mean±SEM. N=5 mice. *P* values were obtained by *t* test. InsR indicates insulin receptor; and IP, immunoprecipitation.

SR-localized Ca^{2+} cycling proteins, such as SERCA2 and RyR2, are crucial for the modulation of cardiac contractility in response to βAR activation.[11] We hypothesize that a population of intracellular β_1ARs are present at the SR. We examined a potential colocalization of overexpressed β_1AR with SERCA2 or RyR2. β_1AR displayed specific colocalization with SERCA2, but not RyR2, in mouse AVMs (Figure 1C through 1E and Figure IIA in the Data Supplement). As controls, overexpressed β_2AR did not display overlap with either SERCA2 or RyR2 (Figure IIIA and IIIB in the Data Supplement). Next, we applied proximity ligation assay to further explore the proximal relationship between β_1AR and SERCA2. We detected robust proximity ligation assay signals in mouse AVMs costained for β_1AR and SERCA2, suggesting that these β_1ARs and SERCA2 are at ≤40 nm apart. Conversely, proximity ligation assay signals were not detected after costaining with antibodies against β_1AR and IgG or against β_1AR and RyR2 and only a small amount of proximity ligation assay signals was detected after costaining for β_1AR and JP2 (junctophilin 2; Figure 1F and Figure IIB through IID in the Data Supplement). Moreover, endogenous β_1AR was pulled down with β_1AR antibody (Figure 1G). We found that both SERCA2 and its regulatory protein, PLB, were coimmunoprecipitated with the endogenous β_1ARs. In contrast, RyR2 was not observed in the immunoprecipitation. Furthermore, we applied a supersensitive single-molecule pull-down assay[20] to visualize individual β_1AR-SERCA2 complex in heart lysate. Figure 1H shows that β_1AR antibody was able to pull-down a significant amount of SERCA2 molecules compared with control IgG antibody. Together, these data demonstrate that a population of β_1ARs is localized at the SR and forms a complex with SERCA2 in AVMs.

OCT3 Is Required to Activate the Pool of β_1ARs by Catecholamine at the SR

β-adrenoceptor stimulation results in activation of PKA, a key mediator for phosphorylating multiple Ca^{2+} handling proteins (eg, PLB).[21] To directly assess PKA activity induced by the SR-localized β_1AR, we employed a genetically encoded Förster resonance energy transfer–based AKAR (A kinase activity reporter) that is anchored at the intracellular SERCA2 complex on the SR (SR-AKAR3).[17,18] In

comparison, the PKA activity induced by the PM-localized β_1AR was assessed by an AKAR3 anchored on the PM (PM-AKAR3).

Previous studies showed that OCT3-mediated transportations of epinephrine or norepinephrine into cells are essential for activating intracellular adrenergic receptors in cardiomyocytes.[2,8] Here, we used mice with genetic deletion of OCT3 to examine the impacts of OCT3 on subcellular PKA activity induced by PM- or SR-localized β_1AR (PM-β_1AR or SR-β_1AR). Unlike endogenous catecholamines, isoproterenol (Log$P$=1.4) and dobutamine (Log$P$=3.6), 2 membrane-permeant β agonists, activate internal βAR via passive diffusion into myocytes (Figure 2A and 2B).[7] Therefore, deleting OCT3 should selectively block the access of norepinephrine and epinephrine but not isoproterenol and dobutamine to SR-localized β_1AR (Figure 2A and 2B). Norepinephrine, epinephrine, isoproterenol, and dobutamine all induced robust PKA activity at both the PM and SR in WT myocytes (Figure IVA through IVD in the Data Supplement). The kinetics of ligand-induced PKA activity at the PM was comparable but were faster than those at the SR (Figure IVA through IVE in the Data Supplement). Among the SR-PKA activity, the dobutamine-induced increases were faster than those by isoproterenol, epinephrine, and norepinephrine, consistent with its faster membrane permeability (Figure IVA through IVE in the Data Supplement). As predicted, the isoproterenol-induced PKA activity at both the PM and SR was not affected by deletion of OCT3 (Figure 2C through 2D). However, deletion of OCT3 selectively attenuated norepinephrine-induced PKA activity at the SR without affecting PKA activity at the PM (Figure 2E and 2F). Moreover, inhibition of β_1AR with CGP20712a completely abolished PKA activity induced by norepinephrine and isoproterenol at both PM and the SR in AVMs, whereas inhibition of β_2AR with ICI118551 did not affect these PKA activities (Figure IVF through IVI in the Data Supplement). These observations were not due to change in β_1AR expression in OCT3-KO hearts (Figure V in the Data Supplement). Additionally, we have examined PKA activity at the myofilaments with an AKAR3 anchored on the troponin complex (myofilament-AKAR3[18]), which is adjacent to the SR. Our data show deletion of OCT3 selectively attenuated norepinephrine but not isoproterenol-induced PKA activity on the myofilaments (Figure VA in the Data Supplement). To explore specific PKA substrate affected by SR-localized β_1AR activation, we then examined PKA phosphorylation of PLM (phospholemman, Ser^{63}) and LTCC (Ser^{1928}) at the PM, PLB (Ser[16]) and RyR2 (Ser^{2808}) at the SR, TnI (Ser$^{23/24}$), and MYBP-C (Ser^{282}, Ser^{273}, Ser^{302}) at myofilament. Deletion of OCT3 selectively attenuated norepinephrine-induced PKA phosphorylation of PLB and TnI without affecting PKA phosphorylation of PLM, LTCC, RyR2, and MYBP-C (Figure 2G and 2H and Figure VB through VH in the Data Supplement). In comparison, isoproterenol-induced PKA phosphorylation of both PLM and PLB was not affected by OCT3 deficiency (Figure 2G and 2H). These data suggest that deletion of OCT3 blocks catecholamine-dependent activation of the β_1ARs at the SR and the myofilament and downstream PKA-dependent PLB and TnI phosphorylation.
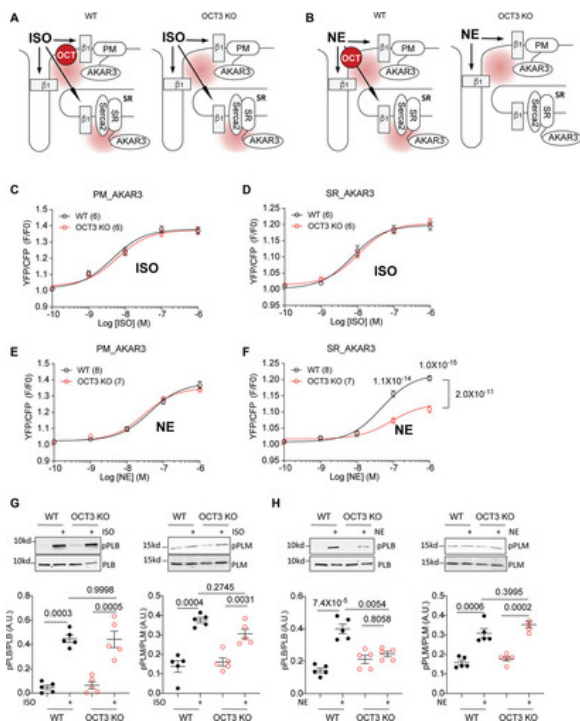
Figure 2. Differential local regulation of norepinephrine (NE)-induced β₁AR (β₁-adrenoceptors)/PKA (protein kinase A) activities in wild-type (WT) and OCT3-KO (organic cation transporter 3 knockout) adult ventricular cardiomyocytes (AVMs). A and B, Schematics of local activation of β₁AR-induced PKA activity at subcellular locations and detection using Förster resonance energy transfer (FRET) based biosensors (plasma membrane, plasma membrane (PM)-AKAR (A kinase activity reporter) 3 and sarcoplasmic reticulum [SR]-AKAR3) in WT and OCT3-KO AVMs. Isoproterenol (ISO), norepinephrine (NE). FRET assay was analyzed with F/F0 of emission ratio of YFP (527nm) to CFP (476nm), YFP/CFP ratio. **C and D,** Concentration-response curves of changes in YFP/CFP ratio after ISO stimulation in WT and OCT3-KO AVMs expressing PM-AKAR3 or SR-AKAR3. Data were from 6 WT and 6 OCT3-KO mice. **E and F,** Concentration-response curves of changes in YFP/CFP ratio after NE stimulation in WT and OCT3-KO AVMs expressing PM-AKAR3 or SR-AKAR3. Data were from 8 WT and 7 OCT3-KO mice. Data are shown in mean±SEM. *P* values were obtained by 2-way ANOVA with Tukey multiple comparison test when comparing WT to OCT3-KO. **G and H,** Detection of pPLB (phosphorylation of phospholamban) at serine 16 and pPLM at serine 63 in mouse AVMs after stimulation with 100 nmol/L ISO or 100 nmol/L NE. Data are shown in mean±SEM (N=5). AU (arbitrary unit) is defined as the ratio of intensity of phosphorylated proteins over intensity of total proteins. *P* values were obtained by 2-way ANOVA with Tukey multiple comparison test. A.U indicates arbitrary unit; and SERCA2, sarcoplasmic endoplasmic reticulum Ca²⁺-ATPase 2.

Functional Consequences of the Internal Pool of β₁ARs on Adult Ventricular Myocyte

We then assessed whether the deletion of OCT3 affects catecholamine-induced Ca²⁺ handling and sarcomere shortening in isolated AVMs. Compared with basal conditions, stimulation with different βAR agonists (isoproterenol, dobutamine, norepinephrine, and epinephrine) led to increased sarcomere shortening and Ca²⁺ transient amplitude and decreased Ca²⁺ decay tau in both WT and OCT3-KO AVMs (Figure 3A through 3H and Figure VIA through VIK in the Data Supplement). Dobutamine induced a faster inotropic response than isoproterenol and norepinephrine in WT AVMs. The increases in excitation-contraction coupling were blocked by β₁AR antagonist CGP20712a but not affected by β₂AR inhibition with ICI118551 (Figure VII in the Data Supplement). Deletion of OCT3 did not affect isoproterenol- or dobutamine-induced inotropic responses (Figure 3A through 3D and Figure VID through VIK in the Data Supplement). Conversely, the norepinephrine- and epinephrine-promoted

responses in contraction, Ca^{2+} transients, and $[Ca^{2+}]_i$ decay tau were significantly attenuated in OCT3-KO versus WT AVMs (Figure 3E through 3H and Figure VIJ through VIK in the Data Supplement). Deletion of OCT3 also shifted the norepinephrine dose-response curve of sarcomere shortening to the right without affecting isoproterenol-induced dose-response curve (Figure 3I and 3J).
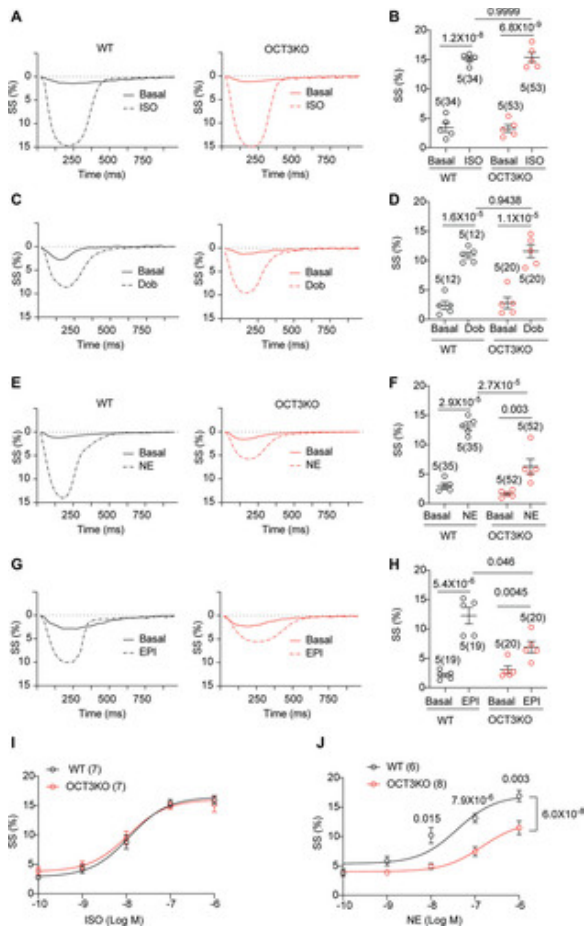
Figure 3. Deletion of OCT3 (organic cation transporter 3) attenuates stimulation of myocyte contractility with norepinephrine (NE) and epinephrine (EPI).A and **B**, Representative traces show sarcomere shortening (SS) before (close line) and after (dash line) stimulation with isoproterenol (ISO; 100 nmol/L) in the wild-type (WT) and OCT3-knockout (KO) mouse adult ventricular cardiomyocytes (AVMs). The peak SS were quantified. **C** and **D**, Representative traces show fractional shortening before (close line) and after (dash line) the application of dobutamine (Dob, 1μmol/L) in WT and OCT3-KO AVMs. The peak SS were quantified. **E** and **F**, Representative traces show SS before (close line) and after (dash line) the application of NE (100 nmol/L) in WT and OCT3-KO AVMs. The peak SS were quantified. **G** and **H**, Representative traces show SS before (close line) and after (dash line) the application of EPI (epinephrine,100 nmol/L) in WT and OCT3-KO AVMs. The peak SS were quantified. For **A–H**, data are shown in mean±SEM. AVM (in the parenthesis) and animal numbers are indicated. *P* values were obtained by 2-way ANOVA analysis followed by Tukey multiple comparison test. **I** and **J**, Dose-response curves of SS in AVMs after stimulation with ISO (7 WT and 7 OCT3-KO mice) or NE (6 WT and 8 OCT3-KO mice). *P* values were obtained by 2-way ANOVA with Tukey multiple comparison test when comparing OCT3-KO to WT. SERCA2 indicates sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase 2.

To independently test the role of OCT3 in the SR-located β₁AR/PKA signaling, we applied an OCT3/EMT inhibitor corticosterone to prevent norepinephrine transport into myocytes (Figure 4A and 4B). While corticosterone had minimal effect on norepinephrine-induced PKA activity at the PM domain, it

significantly attenuated norepinephrine-induced PKA activity at the SR domain (Figure 4C and 4D). Corticosterone did not change isoproterenol-induced PKA activity at either the PM or SR domain in rat AVM (Figure 4C and 4D). In agreement, corticosterone significantly reduced norepinephrine- but not isoproterenol-induced PKA phosphorylation of PLB at Ser[16] (Figure 4E and 4F). Corticosterone also significantly attenuated norepinephrine-induced increases in contractility, Ca^{2+} transient amplitude, and rate of Ca^{2+} transient decay (Figure 4G through 4I). However, these corticosterone effects were absent in the responses to isoproterenol stimulation (Figure 4J through 4L). Together, these data suggest that OCT3-dependent transport is required for stimulation of SR-localized $β_1AR$/PKA/PLB signal cascade for optimal increases in Ca^{2+} handling and contractility.
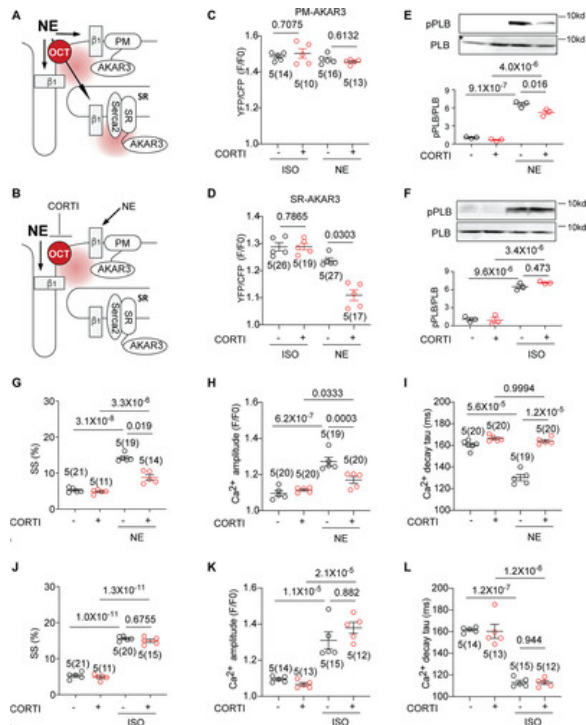

Figure 4. Inhibition of organic catecholamine transporters reduces norepinephrine (NE)-promoted sarcoplasmic reticulum (SR)-localized $β_1AR$ ($β_1$-adrenoceptors) signal and myocardial contractility.A and **B**, Schematics show detection of $β_1AR$-induced PKA (protein kinase A) activity at different subcellular locations with and without OCT3 (organic cation transporter 3) inhibitor corticosterone (CORTI). **C** and **D**, Rat adult ventricular cardiomyocytes (AVMs) expressing plasma membrane (PM)-AKAR (A kinase activity reporter) 3 or SR-AKAR3 Förster resonance energy transfer (FRET) biosensor were pretreated with CORTI (corticosterone,1 µmol/L) before stimulation with NE (100 nmol/L) or isoproterenol (ISO; 100 nmol/L). FRET was analyzed as F/F0 of YFP/CFP ratio. Data show the maximal increases in YFP/CFP ratios in mean±SEM. AVM (in the parenthesis) and rat numbers are indicated. *P* values were obtained by 1-way ANOVA with Tukey multiple comparison test. **E** and **F**, Representative immunoblot detection and quantification of phosphorylated serine 16 (pPLB) and total PLB (phospholamban) in rat AVMs. Cells were treated with 5-minute incubation with ISO (100 nmol/L) or NE (100 nmol/L) in the absence and presence of CORTI pretreatment. AU (arbitrary units) is defined as the ratio of intensity of phosphorylated proteins over intensity of total proteins. Data are shown in mean±SEM (N=3). *P* values were obtained by 1-way ANOVA with Tukey multiple comparison test. **G–L**, Rat AVMs were loaded with Ca^{2+} indicator, 5 µmol/L Fluo-4 AM, and pretreated with CORTI (1 µmol/L) before stimulation with NE (100 nmol/L) or ISO (100 nmol/L). Sarcomere shortening (SS) and calcium transient amplitude and tau were recorded with 1 Hz pacing. Data are shown in mean±SEM. AVM (in the parenthesis) and animal numbers are indicated in figures. *P* values were obtained by 1-way ANOVA with Tukey multiple comparison test.

Activation of the Internal β$_1$ARs at the SR Was not Blocked by Membrane-Impermeant β-Blockers

Sotalol, a membrane-impermeant β-blocker (Log$P$=0.24), has limited access to intracellular β$_1$AR.[22] To further dissect the physiological function of the SR-β$_1$AR, we applied sotalol to selectively block the PM-β$_1$AR but permit access and activation of SR-β$_1$AR by agonists. In contrast, propranolol, a membrane-permeant β-blocker (Log$P$=3.48), is expected to block βARs both on the PM and at intracellular membranes (Figure VIIIA in the Data Supplement). We tested the accessibilities of sotalol and propranolol to the β$_1$ARs by examining their abilities to compete with fluorescent FL-Prop from binding to β$_1$ARs. Confocal images of FL-Prop fluorescent staining in WT mouse AVMs showed a distribution of βAR on both surface and intracellular membranes (Figure VIIIB in the Data Supplement). Sotalol pretreatment did not block the intracellular staining (Figure VIIIB in the Data Supplement), whereas addition of propranolol effectively competed against FL-Prop from binding to β$_1$AR in AVMs (Figure VIIIB in the Data Supplement). Addition of isoproterenol and norepinephrine also effectively competed against FL-Prop from binding to β$_1$AR in AVMs. Thus, intracellular β$_1$AR can be blocked by membrane-permeant β-blockers but are less accessible to membrane-impermeant β-blockers.

Sotalol was then applied to isolate PKA activity induced by local compartmentalized β$_1$AR at the SR (Figure 5A through 5C). In WT, β$_1$AR-KO, and β$_2$AR-KO mouse neonatal myocytes and WT mouse, rat, and rabbit AVMs, sotalol readily inhibited isoproterenol-induced and β$_1$AR mediated PKA activity at the PM but not those at the SR (Figure 5D through 5G, Figures IXA through IXD and Figure X in the Data Supplement). In rat AVMs, the IC$_{50}$ of sotalol for inhibition of isoproterenol-induced PKA activity at the SR was about 10-fold higher than that of PKA activity at the PM (Figure 5G, PM-AKAR3 IC$_{50}$=2.22±0.06 μmol/L; SR-AKAR3 IC$_{50}$=21.5±0.06 μmol/L). In contrast, the membrane-permeant β-blocker propranolol inhibited isoproterenol-induced PKA activity at both the PM and SR in a concentration-dependent manner (Figure 5F, PM-AKAR3 IC$_{50}$=74.0±0.05 nmol/L, SR-AKAR3 IC$_{50}$=99.2±0.06 nmol/L). Another poorly permeant β-blocker, atenolol (Log$P$=0.6), acted similarly to sotalol by selectively inhibiting isoproterenol-induced PKA activity only at the PM in rat AVMs (Figure IXE and IXF in the Data Supplement). In comparison, membrane-permeant carvedilol (Log$P$=4.19) inhibited isoproterenol-induced PKA activity at both the PM and the SR (Figure IXE and IXF in the Data Supplement). Together, these data support that local activation of SR-β$_1$AR-PKA signaling was minimally suppressed by membrane-impermeant β-blockers, a conserved mechanism throughout different cardiac myocytes from different species. This also indicates that the β$_1$AR at the SR is not activated at the PM and then translocated to the SR.
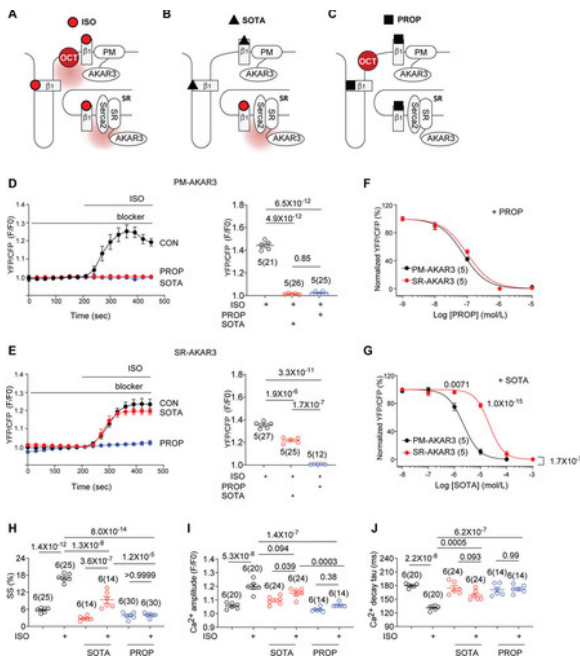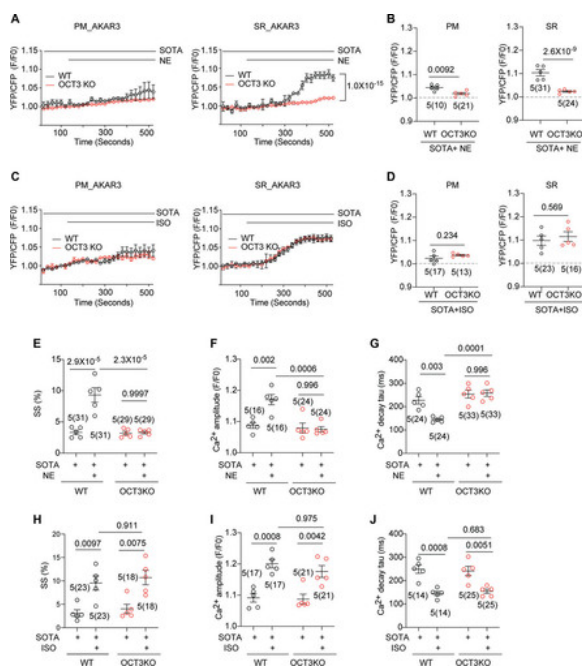
Figure 5. Activation of β₁AR (β₁ -adrenoceptors) in the sarcoplasmic reticulum (SR) is essential for maximal stimulated contractility in adult ventricular cardiomyocytes (AVMs).A–C, Schematics show detection of β₁AR-induced PKA (protein kinase A) activity at different subcellular locations (plasma membrane [PM] and SR) with Förster resonance energy transfer (FRET) based biosensors in the presence of membrane-impermeant β-blocker sotalol (SOTA) or the membrane-permeant β-blocker propranolol (PROP). **D** and **E**, Rat AVMs expressing PM-AKAR (A kinase activity reporter) 3 or SR-AKAR3 were stimulated with 100 nmol/L isoproterenol (ISO) with 5-min pretreatment of 25 µmol/L SOTA (blue) or 1 µmol/L PROP (red) or without pretreament (CON). Representative time courses show FRET response of PM-AKAR3 or SR-AKAR3 in AVMs. FRET was analyzed as F/F0 of YFP/CFP ratio. Data are shown in mean±SEM. AVM (in the parenthesis) and rat numbers are indicated. *P* values were obtained by 1-way ANOVA analysis with Tukey multiple comparison test. **F** and **G**, Dose-dependent inhibition curves of PROP or SOTA on ISO-induced increases in FRET responses in AVMs expressing PM-AKAR3 or SR-AKAR3. Data show YFP/CFP ratios normalized against the maximal increases induced by ISO in the absence of β-blocker (N=5 rats). *P* values were obtained by 2-way ANOVA analysis followed by Tukey multiple comparison test when compared with OCT3-KO (organic cation transporter 3 knockout) (PM-AKAR3: PROP, IC₅₀=7.399×10⁻⁸ mol/L, SR-AKAR3: PROP, IC₅₀=9.92×10⁻⁸ mol/L; PM-AKAR3: SOTA, IC₅₀=2.216×10⁻⁶ mol/L; SR-AKAR3, STOA, IC₅₀=2.149×10⁻⁵ mol/L). **H–J**, Rat AVMs were incubated with Ca²⁺ indicator (5 µmol/L Fluo-4 AM) before 1 Hz pacing. After pretreatment with SOTA (25 µmol/L) or PROP (1 µmol/L), sarcomere shortening (SS) and calcium transient were recorded before and after stimulation with 100 nmol/L ISO. The peak SS, amplitude of Ca²⁺ transient, and rate of Ca²⁺ decay (Tau) are shown as mean±SEM. Animal numbers and cell numbers are indicated in figures. *P* values were obtained by 1-way ANOVA analysis with Tukey multiple comparison test. SERCA2 indicates sarcoplasmic endoplasmic reticulum Ca²⁺-ATPase 2.

Activation of the β₁AR at the SR Promotes Optimal Cardiac Contractility and Ca²⁺ Handling in AVMs

Consistent with the PKA activity detected at the SR, sotalol blocked isoproterenol-induced PKA phosphorylation of LTCC at Ser[1928] but did not affect isoproterenol-induced PKA phosphorylation of PLB at Ser[16] in mouse, rat, or rabbit AVMs (Figure XI in the Data Supplement). However, propranolol abolished isoproterenol-induced phosphorylation of PLB and LTCC (Figure XI in the Data Supplement). These data support that activation of the SR-β₁AR signaling promotes SR-localized PKA phosphorylation of PLB in AVMs. Accordingly, isoproterenol-induced increases in sarcomere shortening, Ca²⁺ transient amplitude, and decreases in tau were partially reduced by sotalol but completely abolished by propranolol in rat AVMs (Figure 5H through 5J). These data suggest that phosphorylation of PLB by PKA is mediated by localized β₁AR signaling at the SR and promotes myocyte contractility.

Similarly, sotalol selectively inhibited norepinephrine-induced PKA activity at the PM yet permitted increases in PKA activity in the SR local domain (Figure 6A through 6D and Figure XIIA through XIIF in the Data Supplement). We then assessed whether OCT3 controls the norepinephrine-induced local PKA activity at the SR in the presence of sotalol. Deletion of OCT3 abolished PKA activity at the SR domain induced by norepinephrine in the presence of sotalol (Figure 6A and 6B and Figure XIIA through XIIF in the Data Supplement). In contrast, isoproterenol-induced PKA activity at the SR was not affected by OCT3-KO and sotalol pretreatment (Figure 6C and 6D). In the presence of sotalol, deleting OCT3 completely blocked norepinephrine-induced increases in sarcomere shortening, Ca²⁺transient, and decreases in Ca²⁺ decay tau (Figure 6E through 6G) but did not affect the responses induced by isoproterenol (Figure 6H through 6J). These data show that in the presence of membrane-impermeant β-blocker sotalol, norepinephrine can selectively stimulate SR-β₁AR signaling to promote local PKA activity and enhance myocyte contractility. These activities can be blocked by deletion of OCT3 that is responsible for uptake of norepinephrine into AVMs.


Figure 6. Activation of β₁AR (β₁-adrenoceptors) at the sarcoplasmic reticulum (SR) promotes myocyte calcium cycling and contractility.A and B, Wild-type (WT) and OCT3-KO (organic cation transporter 3 knockout) adult

ventricular cardiomyocytes (AVMs) were used to express plasma membrane (PM)-AKAR (A kinase activity reporter) 3 or SR-AKAR3 biosensors. Förster resonance energy transfer (FRET) was analyzed as F/F0 of YFP/CFP ratio. Representative curves and maximal changes show subcellular PKA (protein kinase A) FRET responses to norepinephrine (NE; 100 nmol/L) stimulation in AVMs pretreated with sotalol (SOTA, 25 μmol/L). Animal numbers and cell numbers (in the parenthesis) are indicated. *P* values were obtained by 2-way ANOVA analysis followed by Tukey multiple comparison test when compared to OCT3-KO. **C** and **D**, WT and OCT3-KO mouse AVMs were used to express PM-AKAR3 or SR-AKAR3 biosensors. Representative curves and maximal changes show subcellular PKA FRET responses to isoproterenol (ISO; 100 nmol/L) stimulation in AVMs pretreated with sotalol (SOTA, 25 μmol/L). Animal numbers and cell numbers (in the parenthesis) are indicated. *P* values were obtained by 2-way ANOVA analysis followed by Tukey multiple comparison test when compared to OCT3-KO. **E–G**, AVMs from WT and OCT3 hearts were stimulated with NE (100 nmol/L) in the presence of SOTA (25 μmol/L) pretreatment. Data show maximal changes in sarcomere shortening (SS), Ca^{2+} transient amplitude, and rate of Ca^{2+} decay (Tau) as mean±SEM. Animal numbers and cell numbers (in the parenthesis) are indicated in figures. *P* values were obtained by 2-way ANOVA analysis with Tukey multiple comparison test. **H–J**, AVMs from WT and OCT3 hearts were stimulated with ISO (100 nmol/L) in the presence of SOTA (25 μmol/L) pretreatment. Data show maximal changes in SS, Ca^{2+} transient amplitude, and Tau as mean±SEM. Animal numbers and cell numbers (in the parenthesis) are indicated. *P* values were obtained by 2-way ANOVA analysis with Tukey multiple comparison test.

OCT3 Deficiency Impairs Catecholamine Transport in Heart and Attenuates Epinephrine-Induced Inotropy and Heart Rate In Vivo

Finally, we sought to understand the impacts of intracellular β_1AR activation on adrenergic regulation of cardiac function in vivo. We observed no differences in heart weight/body weight ratios of OCT3-KO and WT mice (Figure 7A), indicating a grossly normal cardiac structure in OCT3-KO hearts. However, deletion of OCT3 reduced endogenous norepinephrine concentration in hearts compared with WT controls, whereas OCT3-KO and WT mice had comparable norepinephrine concentrations in the plasma (Figure 7B and 7C). Consequently, OCT3-KO mice show lower cAMP concentrations in hearts relative to WT controls (Figure 7D). These data indicate disrupted catecholamine transport and potential intracellular adrenergic receptor activation by OCT3 deletion. Compared with WT, OCT3-KO mice showed no difference in cardiac function, including ejection fraction and heart rate at baseline conditions (Figure 7E through 7I). These observations obtained by echocardiography are probably relevant to adaptive gene expressions in myocardium due to OCT3 deletion. The expression of PLB and PLB/SERCA2 ratio were decreased whereas expression of RyR2 was increased in OCT3-KO hearts (Figure XIIIA and XIIIB in the Data Supplement). Furthermore, we detected cardiac β-adrenergic response with 10 microg/kg epinephrine or isoproterenol injection in vivo. Compared with WT, OCT3-KO mice showed no difference in cardiac function after 10 microg/kg isoproterenol administration (Figure 7G through 7I). However, OCT3-KO displayed significantly blunted ejection fraction and heart rate responses to epinephrine injection relative to WT (Figure 7G through 7I). These data show that OCT3 is essential for enhancing myocardial contractility induced by epinephrine in vivo.
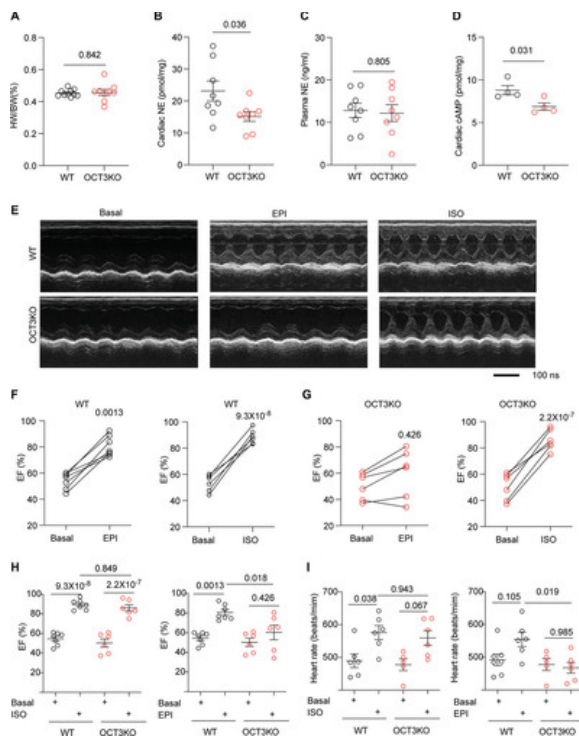
Figure 7. Deletion of OCT3 (organic cation transporter 3) reduces catecholamine uptake, cAMP (cyclic adenosine monophosphate) signal, and inotropic response in response to adrenergic stimulation of mouse hearts. A, Heart weight/body weight ratio (HW/BW %) in wild-type (WT) and OCT3-knockout (KO) mice. Data are shown as mean±SEM (N=8). *P* values were obtained by Student *t* test. **B** and **C**, Quantitative determination of endogenous norepinephrine (NE) in cardiac tissues and the plasma. Data are shown as mean±SEM (N=8). *P* values were obtained by Student *t* test. **D**, Quantitative determination of cAMP levels in cardiac tissues. Data are shown as mean±SEM (N=4). *P* values were obtained by Student *t* test. **E**, Representative echocardiographic images of WT (N=7) and OCT3-KO (N=6) mice at baseline and after intraperitoneal injection of 10 μg/kg isoproterenol (ISO) or epinephrine (EPI). **F–I**, Cardiac ejection fraction (EF) of individual mice before and after intraperitoneal injection of ISO or EPI in WT (N=7) and OCT3-KO (N=6). Quantification of EF and heart rate (HR) in WT and OCT3-KO mice before and after injection with ISO or EPI. Data are shown as mean±SEM. *P* values were obtained by 2-way ANOVA analysis with Tukey multiple comparison test.

Discussion

Adrenergic signaling is one of the most important mechanisms for regulating cardiac function and is typically blunted/disturbed in cardiac diseases including HF.[23] In this study, we provide evidence to support the presence of a pool of β_1ARs at the SR that is essential for enhancing contractility in cardiomyocytes. We also show that monoamine transporter OCT3/EMT–mediated catecholamine uptake is essential for activation of the SR-β_1AR and for promoting phosphorylation of PLB by PKA and cardiac contractility. Furthermore, our data suggest that different clinically used β-blockers differ significantly in their ability to suppress internal β_1AR-PKA signaling due to different membrane permeability. Although membrane-permeant β blockers, such as propranolol and carvedilol, are able to block activation of intracellular β_1ARs, membrane-impermeant β-blockers, such as sotalol and atenolol, cannot readily access intracellular β_1AR. Therefore, this study opens a novel avenue to optimize therapeutic strategies for targeting cardiac β_1 adrenergic signaling in different clinical settings.

Intracellular Adrenergic Receptor Signaling at the SR

Accumulating evidence suggests the presence of GPCRs and G proteins inside mammalian cells.[24] Increasing number of intracellular GPCRs have been identified in animal hearts, including angiotensin[3] and adrenergic receptors.[2,8] In cardiomyocytes, previous studies have characterized both β_1 and α_1ARs but not β_2AR on the nuclear envelope.[1,2] β_1ARs were also found to reside in the Golgi apparatus and initiate an internal G protein-stimulated cAMP signal from the Golgi apparatus, and this internal signal is independent of receptors at the PM.[7,8] However, whether ARs are localized at other subcellular locations and their functional implications in cardiac contractility remain to be elucidated. Here, we used a combination of sophisticated molecular probes, genetically modified mice, and pharmacological inhibitors to uncover important details of the intracellular β_1AR-cAMP-PKA pathway at the SR. We provide solid evidence of the presence of intracellular β_1ARs at the SR in cardiomyocytes, which is associated with SERCA2 and PLB, consistent with a recent report that exogenously expressed β_1ARs bind to SERCA2 in fibroblasts.[25] Meanwhile, our data do not support the presence of a cardiac β_2AR-SERCA2 complex at the SR membrane. This identification of SR-localized β_1ARs provides a novel possibility to explain precisely compartmentalized β_1AR signaling in cardiomyocytes. Upon βAR activation, the locally anchored PKA is activated and promotes phosphorylation of targets within a nanodomain, including LTCC at the PM, PLB on the SR, and troponin I and myosin binding protein C on the myofilaments.[12,18,26] The present work shows that activation of the intracellular β_1ARs at the SR is necessary for promoting local PKA activity to phosphorylate PLB and TnI in cardiomyocytes and probably plays a more critical role in rate-limiting cardiac outputs during the physiological fight-or-flight responses. The intracellular β_1AR and PKA activity was essential to optimize enhancement of Ca^{2+} transients and cardiac contractility. Yet, much remains to be learnt about the composition, regulation, and function of this emerging β_1AR signaling machinery at the SR in hearts.

Requirement of Catecholamine Uptake for Intracellular β_1ARs Activation

Based on lipophilicity, different βARs ligands access intracellular β_1AR either by passive diffusion or transporter-mediated uptake. For instance, the current and prior studies show that hydrophilic catecholamine norepinephrine and epinephrine require OCT3/EMT for cellular uptake,[2] whereas hydrophobic ligands (eg, isoproterenol) can passively cross the PM.[7,8] During sympathetic stimulation, catecholamine is released from nerve termini to stimulate adrenergic receptors on cardiomyocytes. Extracellular catecholamines are quickly reduced mainly by reuptake at nerve termini.[27] However, cardiomyocytes can also take up a portion of catecholamines via OCT3/EMT.[28] A key question is raised by these observations: what is the function of the catecholamine uptake into cardiomyocytes? Traditionally, it is considered as a mechanism to clear excess catecholamines to prevent overstimulation of cardiac adrenergic signaling.[14] However, recent research revealed that intracellular norepinephrine activates Golgi-localized β_1ARs to promote PI4P hydrolysis.[8] In this work, for the first time, we show that inhibition of norepinephrine uptake by OCT3/EMT reduces intracellular PKA activity at the SR as well as contractility in cardiomyocytes. Accordingly, OCT3/EMT dependent-catecholamine uptake is essential for optimal βAR-induced enhancement of contractility in cardiac muscles. Deletion of OCT3 attenuated epinephrine-induced increases in cardiac ejection fraction. Meanwhile, deletion of OCT3 also blunted epinephrine-induced increases in cardiac heart rate, supporting a notion that SR Ca^{2+} content contributes to sinoatrial nodal pacemaker cells for physiological heart rate increases.[29] Further studies may be merited to address impacts of intracellular β_1ARs activation on SR

Ca^{2+} content and Ca^{2+} handling in myocytes. It also remains to be examined whether other transporters such as OCT2 may affect activation of intracellular β_1ARs in hearts. These observations highlight the transporter-dependent uptake of catecholamines as a potential drug target for clinical applications in a variety of cardiovascular conditions.

Similarly, clinically used β-blockers differ significantly in their membrane permeability. Carvedilol is a commonly used β-blocker in HF therapy, and it can permeate the PM and inhibit β_1AR at both the PM and intracellular membrane compartments. Thus, it may potentially inhibit detrimental effects of chronic activation of the intracellular β_1AR such as nuclear PKA activity for maladaptive gene expression. In comparison, membrane-impermeant β-blockers such as sotalol and atenolol, which should mainly block β_1AR only at the PM,[7,8] are not widely used for HF therapy. Nonetheless, these β-blockers are effective as anti-arrhythmic drugs,[30] probably in part due to their ability to inhibit β_1AR on the PM. Our study thus provides a conceptual platform to evaluate and assess individual β-blockers for more efficacious therapy in different clinical applications.

Integrity of β_1ARs at the Cell Surface and Subcellular Membrane

Recent and previous studies indicate that the activation of intracellular βAR/PKA signal generates localization-specific and substrate-specific effects.[7,8] Membrane localization of βARs and their associated complexes precisely fine-tune βAR activation, which is essential to maintain the cellular function and appropriate responses to extracellular stimuli, such as catecholamines.[16,26] More importantly, we found that the integral regulation of β_1AR at both cell surface and the SR is required to achieve maximal enhancement of cardiac contraction via optimally coordinating PKA-dependent phosphorylation of substrates in multiple loci. Impaired integrity of β_1AR/cAMP/PKA signaling and blunted cardiac contractile response to β-adrenergic stimulation have been implicated in pathological stress conditions.[26] It has been reported that in HF βAR activation-induced PKA activity is significantly inhibited or abolished at the SR and at the myofilaments, but well-preserved at the PM,[12,18] suggesting the dysregulation of intracellular βAR/PKA signal is involved in the pathogenesis of HF. Besides, a large body of studies revealed that in human HF, norepinephrine reuptake was impaired.[31] Although cardiac norepinephrine release by sympathetic nerves was increased in patients with HF, cardiac norepinephrine uptake and cardiac norepinephrine contents were significantly reduced. Accordingly, OCT transporters expression is reduced in dilated cardiomyopathy patients.[15] Those observations are consistent with our findings in OCT3-KO mice that disrupted catecholamine uptake leads to decreased norepinephrine concentrations and cAMP in the myocardium. Meanwhile, OCT3-KO mice show attenuated inotropic response to catecholamines. Taken together, these findings suggest a decreased activation of intracellular β_1AR at the SR due to dysregulated OCT3 may contribute to the decreased PKA activity and phosphorylation of PLB associated with cardiac dysfunction. The integrity β_1ARs at intracellular membranes and their role in development of HF remain to be further explored.

In summary, this study uncovers a novel signaling paradigm in which an SR-localized intracellular β_1AR is functionally crucial in adrenergic stimulation of cardiac contractility. Moreover, the data not only help to broaden our current understanding of the general role of β_1AR signaling in cardiac regulation but also open up a novel platform to evaluate and optimize β-blockers in clinical applications based on their biased properties in selective inhibition of cardiac β_1AR at distinct subcellular locations.

Nonstandard Abbreviations and Acronyms

AKARs	a kinase activity reporters
AR	adrenergic receptors
AVMs	adult ventricle myocytes
EMT	extraneuronal monoamine transporter
FL-Prop	BODIPY-tagged propranolol
GPCRs	G-protein coupled receptors
InsR	insulin receptor
JP2	junctophilin 2
LTCC	L-type calcium channel
MYBP-C	myosin binding protein C
KO	knockout
OCT3	organic cation transporter 3
PKA	protein kinase A
PLB	phospholamban
PLM	phospholemman
PM	plasma membrane
RyR2	ryanodine receptor 2
SERCA2	sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase 2
SR	sarcoplasmic reticulum
TnI	troponin I
WT	wild-type

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Footnotes

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Novelty and Significance

What Is Known?

- β_1ARs (β_1-adrenoceptors) are the major subtype driving cardiac contractility in response to catecholamine stimulation.

- β_1ARs exist in intracellular membranes.

- OCT3 (organic cation transporter 3) mediates norepinephrine entry into cardiomyocytes.

What New Information Does This Article Contribute?

- A pool of β_1ARs are associated with the SERCA2 (sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase 2) complex at the sarcoplasmic reticulum (SR).

- Activation of the SR-localized β_1AR is essential to promote phosphorylation of phospholamban.

- Organic Cation transporter 3 is required for catecholamines (norepinephrine and epinephrine) to enter cells and stimulate the β_1AR at the SR.

- Activation of intracellular SR β_1AR is essential to promote cardiac contractility.

Functional β_1ARs exists at the SR and is critical for PKA (protein kinase A)-mediated phosphorylation of phospholamban and cardiac contractility upon catecholamine stimulation. Activation of these

intracellular β_1ARs requires catecholamine transport via OCT3. For decades, catecholamines have been shown to signal via βARs on the plasma membrane to regulate heart contractile functions. Little is known about the presence and function of intracellular βARs in cardiomyocytes. We show the presence of intracellular β_1AR associated with SERCA2 on the SR. Activation of this SR-localized β_1AR is essential for promoting PKA-dependent phosphorylation of PLB (phospholamban) and optimizing the cardiac contraction response. Intracellular β_1AR activation is regulated by catecholamine uptake via the OCT3 transporter or membrane-permeant β-blockers in cardiomyocytes. These findings provide new mechanistic insight into the composition, localization, and regulation of β_1AR signaling. Targeting intracellular β_1AR and its regulators could have potential therapeutic utility in diseases related to cardiac contractile function.