Dihydropyridine Ca\(^{2+}\) Channel Blockers Increase Cytosolic [Ca\(^{2+}\)] by Activating Ca\(^{2+}\)-sensing Receptors in Pulmonary Arterial Smooth Muscle Cells

Aya Yamamura,* Hisao Yamamura,* Qiang Guo, Adriana M. Zimnicka, Jun Wan, Eun A. Ko, Kimberly A. Smith, Nicole M. Pohl, Shanshan Song, Amy Zeifman, Ayako Makino, Jason X.-J. Yuan

Rationale: An increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) in pulmonary arterial smooth muscle cells (PASMC) is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC proliferation and pulmonary vascular remodeling. The dihydropyridine Ca\(^{2+}\) channel blockers, such as nifedipine, have been used for treatment of idiopathic pulmonary arterial hypertension (IPAH).

Objective: Our previous study demonstrated that the Ca\(^{2+}\)-sensing receptor (CaSR) was upregulated and the extracellular Ca\(^{2+}\)-induced increase in [Ca\(^{2+}\)]\(_{cyt}\) was enhanced in PASMC from patients with IPAH and animals with experimental pulmonary hypertension. Here, we report that the dihydropyridines (eg, nifedipine) increase [Ca\(^{2+}\)]\(_{cyt}\) by activating CaSR in PASMC from IPAH patients (in which CaSR is upregulated), but not in normal PASMC.

Methods and Results: The nifedipine-mediated increase in [Ca\(^{2+}\)]\(_{cyt}\) in IPAH-PASMC was concentration dependent with a half maximal effective concentration of 0.20 µmol/L. Knockdown of CaSR with siRNA in IPAH-PASMC significantly inhibited the nifedipine-induced increase in [Ca\(^{2+}\)]\(_{cyt}\), whereas overexpression of CaSR in normal PASMC conferred the nifedipine-induced rise in [Ca\(^{2+}\)]\(_{cyt}\). Other dihydropyridines, nicardipine and Bay K8644, had similar augmenting effects on the CaSR-mediated increase in [Ca\(^{2+}\)]\(_{cyt}\) in IPAH-PASMC; however, the nondihydropyridine blockers, such as diltiazem and verapamil, had no effect on the CaSR-mediated rise in [Ca\(^{2+}\)]\(_{cyt}\).

Conclusions: The dihydropyridine derivatives increase [Ca\(^{2+}\)]\(_{cyt}\) by potentiating the activity of CaSR in PASMC independently of their blocking (or activating) effect on Ca\(^{2+}\) channels; therefore, it is possible that the use of dihydropyridine Ca\(^{2+}\) channel blockers (eg, nifedipine) to treat IPAH patients with upregulated CaSR in PASMC may exacerbate pulmonary hypertension. (Circ Res. 2013;4:640-650.)

Key Words: calcium channel blocker ■ Ca\(^{2+}\)-sensing receptor ■ nifedipine ■ nicardipine ■ pulmonary hypertension ■ smooth muscle cell

Pulmonary vascular remodeling and sustained pulmonary vasoconstriction greatly contribute to the elevated pulmonary vascular resistance and pulmonary arterial pressure (PAP) in patients with pulmonary arterial hypertension (PAH). An increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) in pulmonary arterial smooth muscle cells (PASMC) is a major trigger for pulmonary vasoconstriction (by triggering PASMC contraction) and a critical stimulus for pulmonary vascular medial hypertrophy (by stimulating PASMC migration and proliferation). Intracellular Ca\(^{2+}\), or cytosolic free Ca\(^{2+}\), is thus an important signaling element that mediates PASMC contraction, migration, and proliferation upon membrane depolarization and activation of membrane receptors by vasoactive and mitogenic factors.

In This Issue, see p 575

By selectively blocking voltage-dependent Ca\(^{2+}\) channels (VDCC) in vascular smooth muscle cells, the dihydropyridine Ca\(^{2+}\) channel blockers (eg, nifedipine, nicardipine) have been used to treat patients with systemic\(^{1,2}\) and pulmonary\(^{3,4}\) hypertension. In 15% to 20% of patients with idiopathic PAH (IPAH), acute administration of nifedipine or other...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>[Ca^{2+}]_{cyt}</th>
<th>cytosolic Ca^{2+} concentration</th>
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<tr>
<td>CaSR</td>
<td>Ca^{2+}-sensing receptor</td>
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<tr>
<td>CTEPH</td>
<td>chronic thromboembolic pulmonary hypertension</td>
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<tr>
<td>GPCR</td>
<td>G protein–coupled receptor</td>
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<tr>
<td>IP_{3}</td>
<td>inositol 1,4,5-trisphosphate</td>
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<tr>
<td>IPAH</td>
<td>idiopathic pulmonary arterial hypertension</td>
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<tr>
<td>MCT</td>
<td>monocrotaline</td>
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<tr>
<td>PASMC</td>
<td>pulmonary arterial smooth muscle cell</td>
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<tr>
<td>ROC</td>
<td>receptor-operated Ca^{2+} channel</td>
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<tr>
<td>RVSP</td>
<td>right ventricular systolic pressure</td>
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<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<td>VDCC</td>
<td>voltage-dependent Ca^{2+} channel</td>
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vasodilators could significantly reduce PAP. In these clinically considered as the vasoreactive patients (or responders), the high dose of Ca^{2+} channel blockers (eg, nifedipine) also has long-term therapeutic and beneficial effects determined by hemodynamics, exercise capacity, and survival rate. It is unclear, however, why the classical dihydropyridine Ca^{2+} channel blockers do not work well in most of the IPAH patients and even cause deterioration of the disease.

The Ca^{2+}-sensing receptor (CaSR) is a G protein–coupled receptor (GPCR) in the plasma membrane that can be activated by extracellular Ca^{2+} (and Mg^{2+}), polyamines (eg, spermine), amino acids, and neomycin. Activation of CaSR sets into motion a complex series of intracellular Ca^{2+} signaling events that may be involved in stimulating PASMC contraction, proliferation, and migration. Like some GPCRs coupled to Gq (eg, endothelin receptors), CaSR activation increases the synthesis of inositol 1,4,5 triphosphate (IP_{3}) and diacylglycerol via phospholipase C. IP_{3} binds to the IP_{3} receptor on the sarcoplasmic reticulum (SR) membrane and releases Ca^{2+} from the SR to the cytosol. Depletion of Ca^{2+} from the SR induces Ca^{2+} entry through store-operated Ca^{2+} channels, also referred to as store-operated Ca^{2+} entry or capacitative Ca^{2+} entry. Diacylglycerol directly activates receptor-operated Ca^{2+} channels (ROC) in the plasma membrane; the Ca^{2+} entry through ROC is termed receptor-operated Ca^{2+} entry. In addition to increasing [Ca^{2+}]_{cyt} via receptor-operated Ca^{2+} entry and store-operated Ca^{2+} entry, the extracellular Ca^{2+}-induced activation of CaSR also activates other signal transduction pathways (eg, Akt/mTOR and mitogen-activated protein kinase/extracellular signal regulated kinase) to induce cell proliferation.

Our recent study indicated that the extracellular Ca^{2+}-induced increase in [Ca^{2+}]_{cyt} by activation of CaSR was enhanced, and the CaSR protein expression was upregulated in IPAH-PASMC compared with normal PASMC. Inhibition of CaSR by siRNA in IPAH-PASMC significantly attenuated the extracellular Ca^{2+}-induced rise in [Ca^{2+}]_{cyt} and markedly inhibited IPAH-PASMC proliferation. Similar to the extracellular Ca^{2+}-induced increase in [Ca^{2+}]_{cyt} was enhanced, and the mRNA and protein expression of CaSR was upregulated in PASMC isolated from rats with monocrotaline (MCT)-induced pulmonary hypertension (MCT-PH) compared with PASMC from control rats. Blockade of CaSR by the calcilytic NPS-2143 inhibited the development of MCT-PH. These observations suggest that upregulated expression and enhanced function of CaSR in PASMC are involved in the development of sustained pulmonary vasoconstriction and pulmonary vascular remodeling in patients with IPAH and animals with experimental PH.

In this study, we examined and compared the effects of dihydropyridine Ca^{2+} channel blockers on the CaSR-mediated increase in [Ca^{2+}]_{cyt} in normal PASMC (with low expression level of CaSR) and IPAH-PASMC (with upregulated expression level of CaSR). The data from this study indicate that the dihydropyridine Ca^{2+} channel blockers (eg, nifedipine, nicardipine), although effectively block VDCC in PASMC, significantly enhance the activity of CaSR that is upregulated in PASMC from patients with IPAH and animals with experimental PH. These observations provide a novel explanation for why the therapeutic effect of nifedipine and other dihydropyridine Ca^{2+} blockers is compromised in many IPAH patients. The data from this study also imply that caution should be exercised when the use of nifedipine and nicardipine is considered for the treatment of IPAH patients with upregulated CaSR in PASMC and the gain-of-function mutations in the CaSR gene.

Methods

Cell Preparation and Culture

Explanted peripheral lung tissues of normal control subjects (2 unsuitable organ donors and 2 chronic obstructive pulmonary disease patients without PH) and patients with IPAH (3 patients diagnosed on the basis of National Institutes of Health IPAH Registry with an averaged mean PAP of 56±5 mm Hg who were nonresponders in response to inhalation of nitric oxide or acute intravenous infusion of prostacyclin and Ca^{2+} channel blocker) were used to isolate human PASMC from small pulmonary arteries (with an outer diameter of 300–500 μm). PASMC were also isolated from endarterectomized tissues of patients with chronic thromboembolic PH (CTEPH, 4 patients with an averaged mean PAP of 39±5 mm Hg). The use of human lung tissues and cells was approved by the University of Illinois at Chicago Institutional Review Board. Human PASMC (passes 5–10) cultured in Medium 199 supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY), 100 U/mL penicillin plus 100 μg/mL streptomycin, 50 μg/mL n-valine (Sigma-Aldrich, St. Louis, MO), and 20 μg/mL endothelial cell growth supplement (BD Biosciences, Franklin Lakes, NJ) at 37°C were used for the experiments.

[Ca^{2+}]_{cyt} Measurement

[Ca^{2+}]_{cyt} in PASMC was analyzed using a ratiometric method described previously. Briefly, PASMC cultured on 25-mm cover slips (Fisher Scientific, Pittsburgh, PA) were incubated in 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid (HEPES)-buffered solution containing 4 μM fura-2 acetoxymethyl ester (fura-2/AM; Invitrogen/Molecular Probes, Eugene, OR) for 60 minutes at room temperature (25°C). The fura-2-loaded cells were placed in a recording chamber on the stage of an invert fluorescent microscope (Eclipse Ti-E; Nikon, Tokyo, Japan) equipped with an objective lens (5 Plan Fluor 20x/0.45 ELWD; Nikon), an electron multiplying charge coupled device camera (Evolve; Photometrics, Tucson, AZ), and the NIS Elements 3.2 software (Nikon). [Ca^{2+}]_{cyt} within a region of interest (5×5 μm) was measured as the ratio of fluorescence intensities (F_{510}/F_{380}) of fura-2 at the rate of every 2 seconds. The HEPES-buffered bath solution had an ionic composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl_{2}, 1.2 mM MgCl_{2}, 14 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with 10 N NaOH. The Ca^{2+}-free bath solution was prepared by adding 1 mM EGTA and replacing CaCl_{2} with equimolar MgCl_{2}. Cells in the recording chamber were continuously superfused with HEPES-buffered solution at a flow rate of 2 mL/min. All experiments were carried out at 32°C.
Transfection of cDNA and siRNA
Cultured PASMC were transiently transfected by electroporation with 2 µg of human CaSR cDNA (constructed into pcDNA3.1(+; Invitrogen), 50 nmol/L of control siRNA or scrambled siRNA (sc-37007; Santa Cruz Biotechnology), or siRNA for CaSR (s2440; Applied Biosystems/Ambion, Austin, TX) using an Amaxa Basic Nucleofector kit (Lonza). Experiments using CaSR-overexpressed and siRNA-transfected cells were performed 48 hours after electroporation.

Drugs
All pharmacological reagents were obtained from Sigma-Aldrich. Dihydropyridine compounds (nifedipine, nicardipine, and Bay K8644) were dissolved in dimethyl sulfoxide to make a stock solution of 10 mM. Diltiazem and verapamil were dissolved in distilled water to make a stock solution of 10 mM. Aliquots of the stock solutions were then dissolved into the HEPES-buffered bath solution at various concentrations on the day when the experiments were performed.

MCT-induced PH
Adult male Sprague–Dawley rats (190–200 g in body weight; Charles River Laboratories) were given a single subcutaneous injection of vehicle (dimethyl sulfoxide) or 60 mg/kg MCT (Sigma-Aldrich) to induce PH. For nifedipine treatment, rats were given intraperitoneal (IP) injections of nifedipine (1 mg/kg, once a day for 2 weeks) either 2 or 4 weeks after MCT injection. For hemodynamic measurements, the animals were anesthetized with an IP injection of ketamine (100 mg/kg) and xylazine (26 mg/kg). Right ventricular pressure was determined with a pressure transducer catheter (SPF869, Millar Instruments, Houston, TX) inserted through the right jugular vein and MPVS Ultra (Millar Instruments) data acquisition system. Data were then recorded and analyzed with AD Instruments Laboratory Chart Pro7.0 software.

Statistical Analysis
Pooled data are shown as the mean±SE. The statistical significance between 2 groups was determined by Student t test. The statistical significance among groups was determined by Scheffé test after 1-way ANOVA. Significant difference is expressed in the figures and figure legends as *P<0.05 or **P<0.01.

Results
To test the effects of dihydropyridines on [Ca\(^{2+}\)]\(_{\text{cyt}}\), we first conducted patch clamp experiments to confirm that nifedipine inhibits, and Bay K8644 activates, VDCC in PASMC. As shown in Figure 1, extracellular application of 1 mM nifedipine significantly decreased whole-cell Ca\(^{2+}\) currents in PASMC, elicited by depolarizing the cells from a holding potential of −70 to +10 mV (Figure 1A), whereas Bay K8644 (10 mM) significantly enhanced the currents (Figure 1B). Consistent with its inhibitory effect on L-type Ca\(^{2+}\) channels, acute application of nifedipine (1 mM/L) also significantly and reversibly inhibited pulmonary vasoconstriction induced by 80 mM K\(^{+}\)-mediated membrane depolarization in isolated pulmonary arterial rings (Figure 1C). These data confirm that nifedipine is a blocker of VDCC, whereas Bay K8644 is an activator of VDCC in PASMC.

Nifedipine Enhances CaSR-mediated Increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) in PASMC From IPAH Patients
To elucidate the effects of dihydropyridines on CaSR-mediated increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\), we first superfused PASMC with Ca\(^{2+}\)-free bath solution (0Ca) for 10 minutes and then with 2.2 mM-containing bath solution (2.2Ca) for 10 to 20 minutes to induce a rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) as a result of the extracellular Ca\(^{2+}\)-mediated activation of CaSR. Then, we applied the dihydropyridines,
nifedipine, nicardipine, and Bay K8644 to cells before the extracellular Ca\(^{2+}\)-induced rise in [Ca\(^{2+}\)\(_{cyt}\)] and compared the effects on the CaSR-mediated increases in [Ca\(^{2+}\)\(_{cyt}\)] in PASMC isolated from normal subjects (normal), patients with IPAH (IPAH), and patients with CTEPH (CTEPH).

In normal PASMC perfused with Ca\(^{2+}\)-free solution (0 mM Ca\(^{2+}\) and 1 mM EGTA) for 10 minutes, restoration of extracellular Ca\(^{2+}\) (to 2.2 mM) had little effect on [Ca\(^{2+}\)\(_{cyt}\)] (Figure 1D, left and 1E, top). In IPAH-PASMC, however, restoration of extracellular Ca\(^{2+}\) induced a transient increase in [Ca\(^{2+}\)\(_{cyt}\)] (Figure 1D, center and 1E, center). In CTEPH-PASMC, restoration of external Ca\(^{2+}\) had negligible effect on [Ca\(^{2+}\)\(_{cyt}\)] (Figure 1D, right and 1E, bottom). In the absence of nifedipine, the extracellular Ca\(^{2+}\)-induced increase in [Ca\(^{2+}\)\(_{cyt}\)] via CaSR activation is composed of a large transient phase and a small plateau phase in IPAH-PASMC (Figure 1D).

Extracellular application of nifedipine (1 \(\mu\)M), a dihydropyridine VDCC channel blocker (Figure 1A), however, significantly enhanced the extracellular Ca\(^{2+}\)-mediated increase in [Ca\(^{2+}\)\(_{cyt}\)] in IPAH-PASMC, but not in normal PASMC and CTEPH-PASMC (Figure 1D–1F). The exclusive enhancement of the extracellular Ca\(^{2+}\)-induced increase in [Ca\(^{2+}\)\(_{cyt}\)] by nifedipine in IPAH-PASMC was potentially owing to the upregulation of CaSR protein expression in these cells compared with PASMC from normal subjects and CTEPH patients. Extracellular application of vehicle (0.1% dimethyl sulfoxide) did not affect the CaSR-mediated increase in [Ca\(^{2+}\)\(_{cyt}\)] in IPAH-PASMC (data not shown). These data indicate that nifedipine significantly enhances the extracellular Ca\(^{2+}\)-mediated increase in [Ca\(^{2+}\)\(_{cyt}\)] in IPAH-PASMC where protein expression of CaSR is upregulated in comparison with normal PASMC. In the next set of experiments, we examined whether nifedipine directly affected the resting [Ca\(^{2+}\)\(_{cyt}\)] in PASMC.

**Nifedipine Directly Increases [Ca\(^{2+}\)\(_{cyt}\)] in PASMC with Upregulated CaSR Expression**

In normal PASMC and CTEPH-PASMC, extracellular application of 1 \(\mu\)M nifedipine had no effect on the resting [Ca\(^{2+}\)\(_{cyt}\)] in the absence or presence of extracellular Ca\(^{2+}\), whereas in IPAH-PASMC with upregulated CaSR, nifedipine significantly increased the resting [Ca\(^{2+}\)\(_{cyt}\)] (Figure 2A and 2B). The resting [Ca\(^{2+}\)\(_{cyt}\)] in IPAH-PASMC was dramatically increased by nifedipine in the presence of extracellular Ca\(^{2+}\) (Figure 2A, center, and 2B and 2C). Even in the absence of extracellular Ca\(^{2+}\), nifedipine caused a slight, but statistically significant, increase in [Ca\(^{2+}\)\(_{cyt}\)] in IPAH-PASMC (Figure 2A, center, and 2B and 2C). The nifedipine-mediated increase in the resting [Ca\(^{2+}\)\(_{cyt}\)] in IPAH-PASMC was dose-dependent (at the range of 0.01–10 \(\mu\)mol/L) with an half maximal effective concentration of 0.21 \(\mu\)mol/L and a Hill coefficient of 1.29 (Figure 3).

These data indicate that nifedipine not only enhances the extracellular Ca\(^{2+}\)-induced increase in [Ca\(^{2+}\)\(_{cyt}\)], but also directly increases the resting [Ca\(^{2+}\)\(_{cyt}\)] in IPAH-PASMC with upregulated CaSR. We speculate that nifedipine may directly activate CaSR to induce the increase in [Ca\(^{2+}\)\(_{cyt}\)] or synergistically activate CaSR with the extracellular Ca\(^{2+}\) to elicit the rise in [Ca\(^{2+}\)\(_{cyt}\)] in IPAH-PASMC.

**Figure 2. Nifedipine increases the resting [Ca\(^{2+}\)\(_{cyt}\)] in idiopathic pulmonary arterial hypertension (IPAH)-pulmonary arterial smooth muscle cells (PASMC) with upregulated Ca\(^{2+}\)-sensing receptor. A and B, Representative traces (A) and pseudocolor images (B) showing changes in [Ca\(^{2+}\)\(_{cyt}\)] in normal (blue), IPAH (red) and chronic thromboembolic pulmonary hypertension (CTEPH) (dark green) PASMC before (Cont), during (Nif) and after application of 1 \(\mu\)M nifedipine (Nif) in the absence (0Ca; left) or presence (2.2Ca) of extracellular Ca\(^{2+}\). C, Summarized data (mean±SE) showing the amplitude of Nif-induced transient and plateau phases of increases in [Ca\(^{2+}\)\(_{cyt}\)] in normal, IPAH, and CTEPH PASMC.

Negligible Effect of Nondihydropyridine Ca\(^{2+}\) Channel Blockers on CaSR-mediated Increase in [Ca\(^{2+}\)\(_{cyt}\)]

In addition to nifedipine, we examined whether other dihydropyridine derivatives affect CaSR-mediated rise in [Ca\(^{2+}\)\(_{cyt}\)].
In IPAH-PASMC, acute application of 1 μM nicardipine, a dihydropyridine Ca²⁺ channel blocker, significantly enhanced the extracellular Ca²⁺-induced increase in [Ca²⁺]_{cyt} (Figure 4A). Surprisingly, the dihydropyridine Ca²⁺ channel activator, Bay K8644 (1 μM), also augmented the extracellular Ca²⁺-induced rise in [Ca²⁺]_{cyt} in IPAH-PASMC (Figure 4B). Next, we examined the effect of nondihydropyridine Ca²⁺ channel blockers on the CaSR-mediated increase in [Ca²⁺]_{cyt}. In IPAH-PASMC, acute application of 10 μM diltiazem, a benzothiazepine Ca²⁺ channel blocker (Figure 4C), or 10 μM verapamil, a phenylalkylamine Ca²⁺ channel blocker (Figure 4D), had no effect on the extracellular Ca²⁺-induced rises in [Ca²⁺]_{cyt}. When IPAH-PASMC were superfused with Ca²⁺-containing solution, both nicardipine (Figure 4E) and Bay K8644 (Figure 4F) significantly increased the resting [Ca²⁺]_{cyt} (Figure 4I); however, neither diltiazem (Figure 4H) nor verapamil (Figure 4H) increased the resting [Ca²⁺]_{cyt} (Figure 4I). These data indicate that (1) the augmenting effect of the dihydropyridine derivatives on CaSR-mediated increase in [Ca²⁺]_{cyt} is independent of their effects (inhibition or activation) on VDCC in PASMC and (2) the augmenting effect on the CaSR-mediated increase in [Ca²⁺]_{cyt} in IPAH-PASMC is selective to the dihydropyridine derivatives.

**CaSR Is Necessary and Sufficient to Mediate the Nifedipine-mediated Increase in [Ca²⁺]_{cyt} in PASMC**

To obtain direct evidence for the involvement of CaSR in the nifedipine-induced increase in [Ca²⁺]_{cyt}, we examined the nifedipine effect in IPAH-PASMC treated with the CaSR-specific siRNA and in normal PASMC transfected with CaSR. As shown in Figure 5, transfection of the CaSR-siRNA into IPAH-PASMC decreased CaSR protein expression level (Figure 5A) and significantly inhibited the nifedipine-mediated increase in the resting [Ca²⁺]_{cyt}, in comparison with control cells transfected with scrambled siRNA (control siRNA) (Figure 5B). The averaged data and histograms of the nifedipine-mediated rise in the resting [Ca²⁺]_{cyt} both show that upregulated CaSR is necessary for the nifedipine-mediated increase in [Ca²⁺]_{cyt} (Figure 5C and 5D). In addition, treatment of IPAH-PASMC with the allosteric CaSR antagonist, NPS-2143, significantly attenuated the nifedipine-mediated rise in [Ca²⁺]_{cyt} (Online Figure I). In normal PASMC, overexpression of CaSR markedly enhanced the protein level of CaSR (Figure 6A) and significantly enhanced the nifedipine-mediated increase in [Ca²⁺]_{cyt}, in comparison with normal cells transiently transfected with an empty vector (Figure 6B–6D). These data indicate that CaSR is necessary and sufficient for the nifedipine-mediated increases in [Ca²⁺]_{cyt} in PASMC.

**Nifedipine Further Increases Right Ventricular Systolic Pressure (RVSP) in Rats With Established PH**

In addition to the upregulation in PASMC from IPAH patients, we found that the mRNA and protein expression of CaSR was also upregulated in PASMC, pulmonary arteries, and lung tissues from animals with MCT-PH (Figure 7A and 7B). In rats with established MCT-PH (2 weeks after initial injection of MCT), IP injection of nifedipine (1 mg/kg, once a day for 2 weeks) further enhanced the RVSP (Figure 7C and 7D). RVSP in MCT-PH rats intraperitoneally injected with nifedipine was significantly higher (P=0.011) than in MCT-PH rats injected with vehicle (Figure 7D). The enhanced RVSP was accompanied by further right ventricular hypertrophy determined by the Fulton index, the ratio of right ventricle/ (left ventricle+septum) (Figure 7E). In rats with more severe MCT-PH (4 weeks after initial injection of MCT), however, the nifedipine-mediated enhancement of RVSP (Figure 7F and G) was not associated with a further increase in RV hypertrophy (Figure 7H). These data indicate that, in established MCT-PH, nifedipine actually exacerbates the pulmonary vascular hemodynamics (ie, further increases RVSP), potentially by stimulating CaSR in PASMC. The blockade effect of nifedipine on VDCC is probably compromised by the activating effect on CaSR that is upregulated in PASMC of animals with established PH.

In animals exposed to chronic hypoxia, many investigators have shown that nifedipine (and other dihydropyridine or nondihydropyridine Ca²⁺ channel blockers) prevents (or significantly attenuates) the development of PH. Chronic hypoxia has been demonstrated to downregulate voltage-gated K⁺ channels causing membrane depolarization that subsequently opens VDCC and increases [Ca²⁺]_{cyt} in PASMC.²³ As by blocking VDCC, nifedipine would inhibit Ca²⁺ influx through VDCC and prevent the development of sustained pulmonary vasoconstriction and pulmonary vascular medoid hypertrophy. Our preliminary data showed that CaSR was upregulated in PASMC from animals with both MCT-PH and hypoxia-induced PH,¹⁴ whereas Zhang et al demonstrated that activation...
of mitochondrial H$_2$O$_2$-sensitized CaSR by extracellular Ca$^{2+}$ is involved in hypoxic pulmonary vasconstriction. It is thus unclear why nifedipine only enhanced the development of PH in MCT-PH rats, but not in hypoxia-induced PH animals.\textsuperscript{27-29}

**Discussion**

The major findings of this study are that dihydropyridine derivatives, regardless of their blocking (nifedipine, nicardipine) or activating (Bay K8644) effect on VDCC, enhanced the extracellular Ca$^{2+}$-induced rise in [Ca$^{2+}$]$_{cyt}$ and increased the resting [Ca$^{2+}$]$_{cyt}$ in PASMC from IPAH patients and MCT-PH rats in which CaSR was upregulated. Knockdown of CaSR by siRNA in IPAH-PASMC significantly attenuated the nifedipine-induced increase in [Ca$^{2+}$]$_{cyt}$, whereas overexpression of CaSR by transiently transfecting the human CaSR gene into normal PASMC augmented the nifedipine-mediated rise in [Ca$^{2+}$]$_{cyt}$. In contrast, nondihydropyridine Ca$^{2+}$ blockers, for example, diltiazem (a benzothiazepine VDCC blocker) and verapamil (a phenylalkylamine VDCC blocker), had no effect on the CaSR-mediated increase in [Ca$^{2+}$]$_{cyt}$ in IPAH-PASMC.

CaSR (also known as GPCR2A) is a GPCR that can be activated by polyvalent cations (Ca$^{2+}$, Mg$^{2+}$, and Gd$^{3+}$), amino acids (phenylalanine and glutamate), endogenous polyamines ( spermine and spermidine), polypeptides (amyloid-$\beta$ peptide), and aminoglycoside antibiotics (neomycin), as well as synthetic pharmacological compounds (eg, R-568 and calcet).\textsuperscript{16-18} CaSR is expressed in parathyroid gland, kidney, bone, gastrointestinal tract, skin, brain, and heart.\textsuperscript{16,30,31} CaSR is also expressed in vascular smooth muscle cells\textsuperscript{33-38} and endothelial cells.\textsuperscript{39,40} Activation of CaSR\textsuperscript{33,34,38} is involved in the
CaSR (Ca2+−sensing receptor) is necessary for the dihydropyridine-mediated increase in [Ca2+]cyt in idiopathic pulmonary arterial hypertension (IPAH)−pulmonary arterial smooth muscle cells (PASMC). A, Western blot analysis of CaSR in IPAH-PASMC treated with control siRNA (Control) and CaSR siRNA in different concentration (10, 20, or 30 pmol). β-Tubulin is used as a control. B, Representative traces showing changes in the resting [Ca2+]cyt, before, during, and after application of 1 μM nifedipine (Nif) in IPAH-PASMC treated with control siRNA or CaSR-siRNA. C, Summarized data (mean±SE) showing the amplitude of Nif-induced increases in [Ca2+]cyt in IPAH-PASMC treated with control siRNA (Cont siRNA) or CaSR-siRNA. **P<0.01 vs Cont. D, Amplitude distributions of the Nif-induced rise in [Ca2+]cyt in IPAH-PASMC treated with Cont siRNA (top) or CaSR-siRNA (bottom).

Figure 5. Ca2+−sensing receptor (CaSR) is necessary for the dihydropyridine-mediated increase in [Ca2+]cyt in idiopathic pulmonary arterial hypertension (IPAH)−pulmonary arterial smooth muscle cells (PASMC). A, Western blot analysis of CaSR in normal PASMC transfected with an empty vector (Vector) and the human CaSR gene (CaSR). β-Tubulin is used as a control. B, Representative traces showing changes in the resting [Ca2+]cyt, before, during, and after application of 1 μM nifedipine (Nif) in normal PASMC transfected with Vector or CaSR. C, Summarized data (mean±SE) showing the amplitude of Nif-induced increases in [Ca2+]cyt in normal PASMC transfected with Vector or CaSR. **P<0.01 vs Vector. D, Amplitude distributions of the Nif-induced increase in [Ca2+]cyt in normal transfected with Vector (top) or CaSR (bottom).

CaSR in vascular smooth muscle cells has been shown to contribute to regulating cell proliferation and apoptosis through the mitogen-activated protein kinase and the phospholipase C cascades. CaSR was significantly upregulated in PASMC from IPAH patients in comparison with normal controls. The upregulated CaSR protein expression was associated with a markedly enhanced Ca2+-induced increase in [Ca2+]cyt in IPAH-PASMC. Because CaSR was upregulated and extracellular Ca2+-mediated increase in [Ca2+]cyt was enhanced in PASMC from IPAH patients and in PASMC from animals with experimental PH, our previous data showed that IP injection of the calcilytic NPS-2143 indeed significantly inhibited the development of PH in animals injected with MCT.

As shown in this study (Figure 1), the nifedipine-induced increase in [Ca2+]cyt only occurred in IPAH-PASMC where CaSR was upregulated and in CaSR-transfected normal PASMC where CaSR was overexpressed. These data suggested that the dihydropyridine compounds (nifedipine, nicardipine, and Bay K8644) can directly activate CaSR. As a GPCR, activated CaSR signals through IP3 and diacylglycerol to increase [Ca2+]cyt. IP3 increases [Ca2+]cyt by stimulating Ca2+ release from the SR to the cytosol via IP3 receptors on the SR membrane, whereas diacylglycerol increases [Ca2+]cyt by augmenting Ca2+ influx through ROC on the plasma membrane. In the absence of extracellular Ca2+, the nifedipine-induced increase in [Ca2+]cyt in IPAH-PASMC was much smaller than we expected (Figure 2), in comparison with other GPCR agonists. This phenomenon is, however, very similar to the response to spermine that directly activates CaSR. The marked decline in the nifedipine-induced increase in [Ca2+]cyt in IPAH-PASMC superfused with Ca2+-free solution indicates that (1) Ca2+ influx is the predominant pathway to increase [Ca2+]cyt on activation of CaSR (by nifedipine or spermine) and (2) CaSR is synergistically activated by extracellular Ca2+ and nifedipine (or spermine) to trigger the increase in [Ca2+]cyt (through Ca2+ release and influx). It has been demonstrated that CaSR is functionally coupled to Ca2+ channels on the plasma membrane.
Yamamura et al  Dihydropyridines Activate Ca\textsuperscript{2+}-Sensing Receptors

Dihydropyridine Ca\textsuperscript{2+} channel blockers (eg, nifedipine and nicardipine) are selective blockers of VDCC which have been widely used for the treatment of cardiovascular diseases, such as hypertension, angina, and arrhythmia.\textsuperscript{33,42–46} Conventional Ca\textsuperscript{2+} channel blockers (ie, nifedipine and diltiazem) that selectively block VDCC in PASMC have been used to effectively treat 15% to 20% of IPAH patients who are considered as pulmonary vasoreactive responders.\textsuperscript{3,4} It is, however, unknown why conventional Ca\textsuperscript{2+} channel blockers (ie, VDCC blockers) are not therapeutically effective for a majority of patients with PAH, especially the patients with severe PH.\textsuperscript{3,4} In future studies, it would be very interesting to investigate the possibility that nifedipine may have the same side effect on systemic arteries if CaSR is upregulated in the vascular smooth muscle of the small and resistance arteries in patients with systemic hypertension.

Figure 7. Intraperitoneal injection of nifedipine in animals with established pulmonary hypertension further increases right ventricular systolic pressure and right ventricular hypertrophy. A and B, Western blot analysis (A) and summarized data (mean±SE; B) of Ca\textsuperscript{2+}-sensing receptor (CaSR) in whole lung tissues isolated from control and monocrotaline-induced pulmonary hypertension (MCT-PH) animals. β-Actin is used as a control. C and D, Representative record of right ventricular pressure (RVP; C) and summarized data (mean±SE) showing the peak value of right ventricular systolic pressure (RVSP; D) in normal control rats (Cont, n=6) and MCT-injected rats (MCT, n=6) that are treated with vehicle or nifedipine (+Nif, 1 mg/kg per day for 2 weeks) 2 weeks after the MCT injection. E, Averaged Fulton index (right ventricle [RV]/left ventricle [LV]+septum [S]) ratio; mean±SE) showing that RV hypertrophy is further increased in MCT-rats treated with nifedipine. F and G, Representative record of RVP (F) and summarized data (mean±SE) showing the peak value of RVSP (G) in normal control rats (Cont, n=6) and MCT-injected rats (MCT, n=6) that are treated with vehicle or nifedipine (+Nif, 1 mg/kg per day for 4 weeks) 4 weeks after the MCT injection. H, Averaged Fulton index [RV/(LV+S) ratio; mean±SE] showing that RV hypertrophy is not further increased in MCT-rats treated with nifedipine 4 weeks after MCT injection.

(eg, transient receptor potential [TRP] channels, TRPC1/C3/C6, TRPV5, and TRPM4/M5) to mediate Ca\textsuperscript{2+} influx.\textsuperscript{33,42–46}
The data from this study show that the dihydropyridine Ca\(^{2+}\) channel blockers (eg, nifedipine and nicardipine) not only block VDCC (which would decrease [Ca\(^{2+}\)]\(_{cyt}\)), but also activate CaSR (which would increase [Ca\(^{2+}\)]\(_{cyt}\)) in PASMC. Importantly, the dose or concentration range at which dihydropyridines block VDCC (100 mM/L to 1 mM/L) in vascular smooth muscle cells\(^{50,51}\) overlaps with the dose range at which dihydropyridines activate CaSR (half maximal effective concentration=0.20 mM/L for nifedipine). It is thus impossible to differentiate these 2 opposite effects of nifedipine (and other dihydropyridine Ca\(^{2+}\) channel blockers) using different concentrations or doses. These observations imply that, in IPAH patients (eg, vasoreactive responders) with downregulated voltage-gated K\(^+\) channels in PASMC\(^{52-54}\) where membrane potential is depolarized and VDCC is opened, the dihydropyridine Ca\(^{2+}\) channel blockers (eg, nifedipine and nicardipine) should be a good therapeutic approach.\(^4\) In IPAH patients with upregulated CaSR (and upregulated TRPC channels) in PASMC,\(^{14,20}\) however, the therapeutic effect of nifedipine and nicardipine (by blocking VDCC) is potentially compromised by the stimulatory effect on CaSR. Our in vivo experiments from this study show that IP injection of nifedipine in rats with established MCT-PH actually exacerbated the pulmonary vascular hemodynamics or further enhanced PAP (determined by RVSP via right heart catheterization) and right ventricular hypertrophy (determined by the Fulton index). For IPAH patients with upregulated CaSR in PASMC or with the gain-of-function mutations in the CASR gene, it may be more appropriate to use nondihydropyridine Ca\(^{2+}\) channel blockers (eg, diltiazem and verapamil) in combination with CaSR antagonists or calcilytics (eg, NPS-2143) and TRPC channel blockers to prevent the further activation of CaSR and progression of the disease.

The stimulatory effect of dihydropyridines on CaSR or CaSR-mediated increase in [Ca\(^{2+}\)]\(_{cyt}\) in PASMC is not correlated to their inhibitory or augmenting effect on VDCC. Both dihydropyridine blockers (nifedipine and nicardipine) and activators (Bay K8644) activate CaSR and enhance CaSR-mediated increases in [Ca\(^{2+}\)]\(_{cyt}\) in IPAH-PASMC where CaSR is upregulated. These results indicate that (1) the dihydropyridine derivative-mediated activation of CaSR is unrelated to the blocking action of VDCC and (2) the specific dihydropyridine structure is somehow related to the activation of CaSR or the enhancement of CaSR-mediated increases in [Ca\(^{2+}\)]\(_{cyt}\) in PASMC. It is possible that CaSR and VDCC are colocalized, and the binding of dihydropyridine blockers to VDCC also activates the adjacent CaSR. In IPAH-PASMC, the enhanced Ca\(^{2+}\) influx via nifedipine-mediated activation of CaSR and subsequent activation of ROC and store-operated Ca\(^{2+}\) channels outweighs the inhibitory effect of nifedipine on L-type VDCC owing to upregulated CaSR, with a net effect to promote increased [Ca\(^{2+}\)]\(_{cyt}\) via Ca\(^{2+}\) influx through ROC and store-operated Ca\(^{2+}\) channels.

In cancer cells, nifedipine and extracellular Ca\(^{2+}\) synergistically activate CaSR and increase [Ca\(^{2+}\)]\(_{cyt}\). The resultant downregulation of the expression of thymidylate synthase and survivin promotes the sensitivity of human colon carcinoma cells and breast cancer cells to fluorouracil, a pyridine analogue, and paclitaxel, a mitotic inhibitor.\(^{55,56}\) Survivin, an antiapoptotic protein that is highly expressed in cancer cells, is also expressed in PASM from patients with IPAH and rats with MCT-PH, but not in PASM from normal subjects and control rats.\(^{57,58}\) In vivo inhibition of survivin by inhalation of an adenovirus carrying a phosphorylation-deficient survivin mutant reverses established MCT-PH, whereas in vitro inhibition of survivin by adenoviral infection of the phosphorylation-deficient survivin mutant reduces PASM proliferation and increases apoptosis.\(^{59}\) It would be interesting to investigate whether nifedipine-mediated activation of CaSR downregulates survivin in PASMC, especially in PASMC from patients with IPAH and animals with experimental PH, to promote PASM apoptosis and to regress established PH.

In summary, the dihydropyridine Ca\(^{2+}\) channel blockers (nifedipine and nicardipine) block VDCC and activate CaSR. In normal PASMC where CaSR expression level is low, the predominant effect of nifedipine is to block VDCC, reduce [Ca\(^{2+}\)]\(_{cyt}\), and cause pulmonary vasodilation (and regression of pulmonary vascular remodeling). In PASMC isolated from patients with IPAH and animals with experimental PH where CaSR is significantly upregulated, nifedipine activates CaSR and raises the resting [Ca\(^{2+}\)]\(_{cyt}\). Although this study has limitations owing to the small number of patient samples used, the results have significant clinical relevance. The nifedipine-induced increase in the resting [Ca\(^{2+}\)]\(_{cyt}\) and enhancement of extracellular Ca\(^{2+}\)-induced rise in [Ca\(^{2+}\)]\(_{cyt}\) would compromise its blockade effect on VDCC in IPAH-PASMC and could lead to more severe PH and right ventricular hypertrophy. Therefore, the use of nondihydropyridine Ca\(^{2+}\) channel blockers (eg, diltiazem and verapamil) in combination with specific CaSR antagonists or calcilytics and TRPC channel blockers may be more appropriate for the treatment of IPAH patients with upregulated CaSR in PASMC (or with the gain-of-function mutations in the CASR gene) to avoid the possibility of exacerbating PH and right ventricular hypertrophy. Furthermore, synthesis of selective blocker of CaSR or identification of specific transcription factors that upregulate CaSR in PASMC would greatly help develop new therapeutic approach for PAH.

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Disclosures

None.

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Sun YH, Li YQ, Feng SL, Pan ZW, Xu CQ, Li TT, Yang BF. Calcium-sensing receptor activation contributed to apoptosis stimulates TRPC6 channel in rat neonatal ventricular myocytes. Biochem Biophys Res Comm. 2010;394:955–961.
Dihydopyridines (eg, nifedipine) activate CaSR resulting in an increase in [Ca^{2+}]_{cyt} in pulmonary vascular remodeling and sustained pulmonary vaso-constriction. Increased cytosolic free Ca^{2+} concentration ([Ca^{2+}]_{cyt}) in pulmonary vascular remodeling and sustained pulmonary vaso-constriction contribute to the development of idiopathic pulmonary arterial hypertension (IPAH).

Increased [Ca^{2+}]_{cyt} in IPAH-PASMC, but not in normal PASMC. Ca^{2+}-sensing receptor (CaSR) is upregulated in IPAH-PASMC, contributing to enhanced Ca^{2+} signaling and excessive PASMC proliferation leading to pulmonary vascular remodeling.

Dihydropyridine Ca^{2+} channel blockers, such as nifedipine, are used to treat patients with IPAH; however, these drugs are only effective in 15% to 20% of patients. Ca^{2+}-sensing receptor (CaSR) is upregulated in IPAH-PASMC, contributing to enhanced Ca^{2+} signaling and excessive PASMC proliferation in IPAH patients.

What New Information Does This Article Contribute?

Dihydropyridines (eg, nifedipine) activate CaSR resulting in an increase in [Ca^{2+}]_{cyt} in IPAH-PASMC, but not in normal PASMC.

The use of dihydropyridine Ca^{2+} channel blockers in IPAH patients with upregulated CaSR in PASMC might exacerbate pulmonary hypertension.

What Is Known?

- Pulmonary vascular remodeling and sustained pulmonary vasocostriction contribute to the development of idiopathic pulmonary arterial hypertension (IPAH).
- Increased cytosolic free Ca^{2+} concentration ([Ca^{2+}]_{cyt}) in pulmonary arterial smooth muscle cells (PASMC) triggers pulmonary vasocostriction and stimulates PASMC proliferation leading to pulmonary vascular remodeling.
- Dihydropyridine Ca^{2+} channel blockers, such as nifedipine, are used to treat patients with IPAH; however, these drugs are only effective in 15% to 20% of patients.
- Ca^{2+}-sensing receptor (CaSR) is upregulated in IPAH-PASMC, contributing to enhanced Ca^{2+} signaling and excessive PASMC proliferation in IPAH patients.

IPAH is a rare, progressive, and fatal disease of unknown pathogenesis. Sustained vasocostriction and vascular remodeling owing to PASMC proliferation are key pathogenic events that lead to early morbidity and mortality. These events have been linked to Ca^{2+} mobilization and signaling in PASMC, but precise therapeutic targets to interrupt these pathways have not been identified. We have previously shown that CaSR is upregulated in PASMC isolated from IPAH patients and contributes to increased [Ca^{2+}]_{cyt}.

In this study, we demonstrate that dihydropyridine Ca^{2+} channel blockers, which are used to treat IPAH patients based on their ability to block voltage-dependent Ca^{2+} channels, activate CaSR, leading to increased [Ca^{2+}]_{cyt}. In contrast, nondihydropyridine Ca^{2+} blockers, for example, diltiazem and verapamil, had no effect on the CaSR-mediated increase in [Ca^{2+}]_{cyt} in IPAH-PASMC. Ca^{2+} channel blockers (ie, nifedipine and diltiazem) have been used to effectively treat a subset of IPAH patients who are considered pulmonary vasoactive responders. However, it is not clear why Ca^{2+} channel blockers are not therapeutically effective in a majority of patients with PAH, especially those with severe pulmonary hypertension. This study shows that dihydropyridine increases [Ca^{2+}]_{cyt} in IPAH-PASMC and that the use of dihydropyridine Ca^{2+} channel blockers could exacerbate pulmonary hypertension in IPAH patients with upregulated CaSR in PASMC.
Dihydropyridine Ca\(^{2+}\) Channel Blockers Increase Cytosolic [Ca\(^{2+}\)] by Activating Ca\(^{2+}\) -sensing Receptors in Pulmonary Arterial Smooth Muscle Cells

Aya Yamamura, Hisao Yamamura, Qiang Guo, Adriana M. Zimnicka, Jun Wan, Eun A. Ko, Kimberly A. Smith, Nicole M. Pohl, Shanshan Song, Amy Zeifman, Ayako Makino and Jason X.-J. Yuan

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Online Figure I

Yamamura et al

Online Figure I. Inhibitory effect of NPS-2143 on nifedipine-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC from IPAH patients.

A: Representative traces showing changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ before, during, and after application of 1 μM nifedipine (Nif) in IPAH-PASMC treated with vehicle (DMSO) or NPS-2143 (30 μM). B: Summarized data (means±SE) showing the amplitude of Nif-induced increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ in IPAH-PASMC treated with vehicle (Cont) or NPS-2143 (NPS). **$P<0.01$ vs. Cont. C: Amplitude distributions of the Nif-induced rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in IPAH-PASMC treated with vehicle (Control, upper panel) or NPS-2143 (lower panel).