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Full Paper

# Rho-associated protein kinase and cyclophilin a are involved in inorganic phosphate-induced calcification signaling in vascular smooth muscle cells

Tatsuya Tsuda [a], Masaki Imanishi [b], Mizuho Oogoshi [c, d], Mitsuhiro Goda [b], Yoshitaka Kihira [e], Yuya Horinouchi [c], Yoshito Zamami [b, f], Keisuke Ishizawa [b, f], Yasumasa Ikeda [c], Ichiro Hashimoto [a], Toshiaki Tamaki [c, g], Yuki Izawa-Ishizawa [c, h, *]

[a] Department of Plastic and Reconstructive Surgery, Institute of Biomedical Sciences, Tokushima University Graduate School, Japan
[b] Department of Pharmacy, Tokushima University Hospital, Japan
[c] Department of Pharmacology, Institute of Biomedical Sciences, Tokushima University Graduate School, Japan
[d] Student Lab, Tokushima University School of Medicine, Japan
[e] Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Japan
[f] Department of Clinical Pharmacology and Therapeutics, Institute of Biomedical Sciences, Tokushima University Graduate School, Japan
[g] Anan Medical Center, Japan
[h] AWA Support Center, Tokushima University, Japan

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ABSTRACT

Arterial calcification, a risk factor of cardiovascular events, develops with differentiation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells. Cyclophilin A (CypA) is a peptidyl-prolyl isomerase involved in cardiovascular diseases such as atherosclerosis and aortic aneurysms, and rho-associated protein kinase (ROCK) is involved in the pathogenesis of vascular calcification. CypA is secreted in a ROCK activity-dependent manner and works as a mitogen via autocrine or paracrine mechanisms in VSMCs. We examined the involvement of the ROCK-CypA axis in VSMC calcification induced by inorganic phosphate (Pi), a potent cell mineralization initiator. We found that Pi stimulated ROCK activity, CypA secretion, extracellular signal-regulated protein kinase (ERK) 1/2 phosphorylation, and runt-related transcription factor 2 expression, resulting in calcium accumulation in rat aortic smooth muscle cells (RASMCs). The ROCK inhibitor Y-27632 significantly suppressed Pi-induced CypA secretion, ERK1/2 phosphorylation, and calcium accumulation. Recombinant CypA was found to be associated with increased calcium accumulation in RASMCs. Based on these results, we suggest that autocrine CypA is mediated by ROCK activity and is involved in Pi-induced ERK1/2 phosphorylation following calcification signaling in RASMCs.



## 1. Introduction

Arterial calcification associated with aging, atherosclerosis, diabetes mellitus, and renal failure is a major risk factor for cardiovascular events.[1,2] Vascular calcification in the medial layer is more relevant than intimal calcification to the complication of diabetes mellitus and past vascular disease history.[1] Medial calcification is thought develop as a result of vascular smooth muscle cell (VSMC) differentiation into osteoblast-like cells, followed by their apoptosis.[3,4] Therefore, clarifying the mechanisms of VSMC differentiation will help in understanding the pathophysiology of vascular calcification.

Rho-associated protein kinase (ROCK) is involved in VSMC contraction, migration, and proliferation, and plays important roles in the pathogenesis of cardiovascular diseases (CVD). Recently, several studies have indicated the involvement of the rho-ROCK pathway in VSMC calcification, with ROCK inhibition suppressing

* Corresponding author. AWA Support Center, Tokushima University, 3-18-15, Kuramoto, Tokushima, 7708503, Japan. Fax: +81 88 633 7472.
*E-mail address:* ishizawa.yuki@tokushima-u.ac.jp (Y. Izawa-Ishizawa).
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inorganic phosphate (Pi)- or high glucose-induced calcification in VSMCs.[3,5] Extracellular-signal regulated kinase (ERK) 1/2 is also known to mediate Pi-induced osteogenic responses via the phosphorylation of runt-related transcription factor 2 (runx2) in VSMCs.[6] However, the involvement of the ROCK-ERK1/2 signaling pathway in VSMC osteoblast-like differentiation and calcification remains unclear.

Cyclophilin A (CypA) is a highly-conserved chaperone protein which possesses peptidyl-prolyl isomerase activity, and contributes to aortic aneurysms,[7] atherosclerosis,[8] and other forms of CVD. In our previous study, we demonstrated that CypA expression is upregulated at the aortic wall in an aneurysm mouse model.[9] CypA works via autocrine or paracrine mechanisms,[10] which are mediated by ROCK activity.[11] Secreted CypA stimulates cell proliferation, senescence, inflammatory responses, and apoptosis in several cell types[7,8,10] via binding to the CypA receptor CD147.[12] Autocrine CypA is involved in osteoblast differentiation[13] and bone metabolism,[14] further supporting its role in CVD.

Based on these findings, we hypothesized that CypA contributes to VSMC osteoblast-like differentiation and vascular calcification. To answer this question, we examined the pathological roles and signaling pathways of ROCK, CypA, and ERK1/2 activities in VSMC calcification induced by inorganic phosphate (Pi), a potent cell mineralization initiator.

## 2. Materials and methods

### 2.1. Ethics statement

This study conformed to the Guide for the Care and Use of Laboratory Animals.[15] All animal procedures were performed in accordance with the guidelines of the Animal Research Committee of the University of Tokushima Graduate School. Protocols were approved by the Tokushima University Institutional Review Board for Animal Protection. Paraffin-embedded human samples were obtained from stored autopsy specimens at the Department of Pathology, Tokushima University, where each autopsy was conducted at the Tokushima University Hospital with consent for research use. The protocol was approved by the Ethics Committee of Tokushima University Hospital in accordance with the Declaration of Helsinki.

### 2.2. Cell culture and treatments

Rat aortic smooth muscle cells (RASMCs) were isolated from male Sprague–Dawley 200–250 g rats as previously reported.[16] These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, MO, USA) with 10% FBS. For each experiment, we used 70–90% confluent cultured cells at 37 °C and 5% $CO_2$. Cells were treated with 0.3 or 3 mM dibasic sodium phosphate (Wako, Osaka, Japan) as inorganic phosphate (Pi) stimulation for each assay. Cells were treated with Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Wako) (0.1, 1, and 10 μM), ERK1/2 inhibitor PD98059 (Santa Cruz Biotechnology Inc., TX, USA) (10 μM), and CypA inhibitor 239836 (Merck, Darmstadt, Germany) (1, 10, 30 nM) 30 minutes prior to Pi stimulation.

### 2.3. Measurement of calcium accumulation

RASMCs were stimulated with Pi or cyclophilin human recombinant A (1–165) (Atgen Co. Ltd., Gyeonggi-do, Korea), which shows more than 96% homology with rattus CypA, for 2 or 8 days, after seeding to a 12-well plate. Calcium accumulated on the dish was eluted during an overnight shake with 1 M HCl. The calcium quantity in the eluted liquid was detected in an MXB (methylxylenol blue) assay using a Calcium-E (Ca-E) Test (Wako), with absorbance measured at 610 nm using a microplate reader.

### 2.4. Measurement of alkaline phosphatase (ALP) activation

RASMCs were stimulated with Pi for 2 or 8 days after seeding to 24-well plate. Cells were lysed using phosphate buffered saline (PBS) including 1% Triton X, and absorbance at 410 nm was measured using a Lab Assay ALP kit (Wako) after a p-nitrophenylphosphate substrate reaction. Protein content was quantified using the Bradford assay.

### 2.5. Von Kossa staining

RASMCs were stimulated with Pi for 8 days after sowing to a 35 mm dish. After being fixed with 4% formalin for 30 minutes, cells were stained for 60 minutes in aqueous silver nitrate under UV lighting. Cells were deoxidized and fixed in 5% sodium thiosulfate aqueous solution, washed with PBS, and filmed using EXILIM (CASIO, Tokyo, Japan).

### 2.6. Animal experiments

BKS.Cg- + $Lepr^{db}$/+$Lepr^{db}$/Jcl mice (db/db mice), as models for type 2 diabetes were purchased from Nippon CLEA (Tokyo, Japan), and maintained with free access to water and food. BKS.Cg-m+/m+/Jcl background mice were used as controls. To allow the formation of vascular lesions, mice were maintained until twelve months of age with food and water containing normal quantities of phosphate and glucose. Mice were then anesthetized with pentobarbital (150 mg/kg i.p.) and aortic sections were removed. After fixing in 4% paraformaldehyde, samples were embedded with paraffin and 5 μm slices were prepared as previously reported.[17]

### 2.7. Western blotting

Proteins from mouse aortas and cultured RASMCs were analyzed via western blotting,[17] using antibodies detecting CypA, runx2 (Abcam, MA, USA), phospho-ERK1/2, total-ERK1/2 (Cell Signaling Technology, MA, USA), phospho-myosin phosphatase targeting subunit (MYPT) 1, total-MYPT1, and β-actin (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) at 1:1000 dilution. Activation of ROCK was evaluated by detecting the percentage of phosphorylated MYPT1, an intrinsic substrate of ROCK, and normalizing to total-MYPT1.

### 2.8. Histological analysis

Prepared mouse aortas were stained with hematoxylin and eosin (H&E) or immunostained using runx2 antibody and CypA antibody.[18] Human samples including normal heart, normal aorta, aorta with plaque, and basilar artery with plaque were treated in a similar fashion. A Dako ChemMate Envision kit/HRP (DAB) Universal (Dako, Glostrup, Denmark) was used for DAB staining.

### 2.9. Enzyme-linked immunosorbent assay (ELISA) for CypA

Conditioned media (CM) were collected and concentrated using the ND Protein Precipitation Kit (National Diagnostics Inc., NC, USA) to measure CypA secretion into CM. Concentrated CM were subjected to ELISA,[19] wherein samples were applied to 96-well plates and solid-phased by overnight incubation at 4 °C. After blocking

with 3% BSA, 0.05% Tween 20, and PBS-T, CypA antibody was added and incubated overnight at 4 °C. ECL Rabbit IgG and HRP-linked whole Ab (GE Healthcare, Bucks, UK) were used as secondary antibodies. TMB (3,3′,5,5′- tetramethylbenzidine) substrate solution as reaction buffer and 1 M sulfuric acid (Wako) as stopping buffer were added, and absorption at 450 nm was measured.

### 2.10. Statistical analysis

All experiments were performed with 3–6 replicates. Results are expressed as mean ± standard deviation. Differences were assessed using Student's *t* test for two comparisons, or a two-way analysis of variance (ANOVA) for multiple comparisons, where $p < 0.05$ was considered to indicate statistical significance.

## 3. Results

### 3.1. Pi stimulation increases ALP activity and calcium accumulation in RASMCs

ALP activity increased after 8 day treatment with 3 mM Pi (Fig. 1A, B), and Von Kossa staining showed brown deposits indicating calcium accumulation (Fig. 1C). Results from the MXB assay also showed a significant increase in calcium accumulation (Fig. 1D). Pi stimulation caused an immediate increase in ERK1/2 phosphorylation, with a peak occurring 10 minutes after stimulation (Fig. 1E). Pretreatment with the ERK1/2 inhibitor PD98059 suppressed Pi-induced ALP activity (Fig. 1F). These findings indicate that Pi-induced RASMC calcification was mediated by ERK1/2 activity.

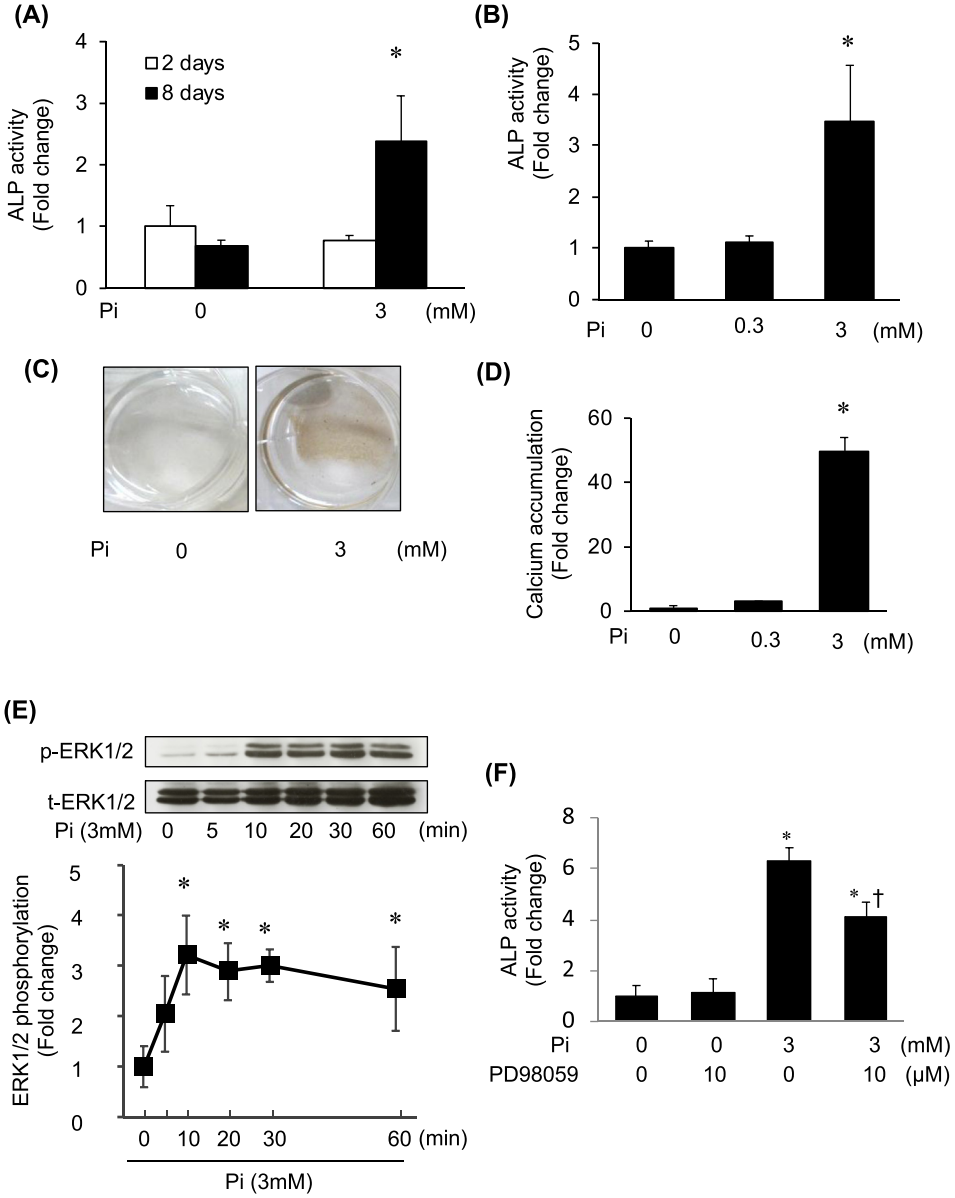


**Fig. 1.** Pi stimulation increases ALP activity and calcium accumulation in RASMCs. (A) ALP activity in RASMCs after stimulation with vehicle or 3 mM Pi for 2 or 8 days. (B) Concentration response of Pi-stimulated ALP activity after 8 days' stimulation. Pi-induced calcium accumulation was visualized by Von Kossa staining (C) and quantified using a Calcium E-test Wako (D) after 8 days' stimulation. (E) RASMCs were stimulated with 3 mM Pi for the indicated time and phosphorylated- and total ERK1/2 expressions were evaluated using western blotting. Upper panels show representative blots and the lower graph shows intensity. (F) Pi-induced ALP activity was measured with or without the ERK1/2 inhibitor PD98059. Values were analyzed using two-way ANOVA for repeated measures. *$P < 0.05$ vs. control, †$P < 0.05$ vs. Pi alone.

### 3.2. Pi stimulates ROCK activity

To examine the involvement of ROCK in Pi-induced calcification signaling, we evaluated phosphorylation of the ROCK substrate, MYPT. MYPT phosphorylation increased immediately and peaked at 10 minutes after Pi stimulation in RASMCs (Fig. 2A), and phosphorylation was inhibited by pretreatment with the ROCK inhibitor Y-27632 in a concentration-dependent manner (Fig. 2B). ERK1/2 phosphorylation was also suppressed by pretreatment with Y-27632 (Fig. 2C), and ROCK inhibition by Y-27632 also significantly suppressed Pi-induced ALP activity and calcium accumulation (Fig. 2D, E). These results indicate that ROCK activity is involved in the Pi-induced calcification pathway by mediating ERK1/2 phosphorylation in RASMCs.

### 3.3. Expressions of runx2 and CypA in aortas from diabetic mice and clinical atherosclerotic plaque samples

Vascular lesions such as atheroma formation could not be observed by H&E staining (Fig. 3B). Immunostaining and Western blot analyses indicated greater expression of runx2 and CypA in aortas from db/db mice compared with control mice (Fig. 3A–C),

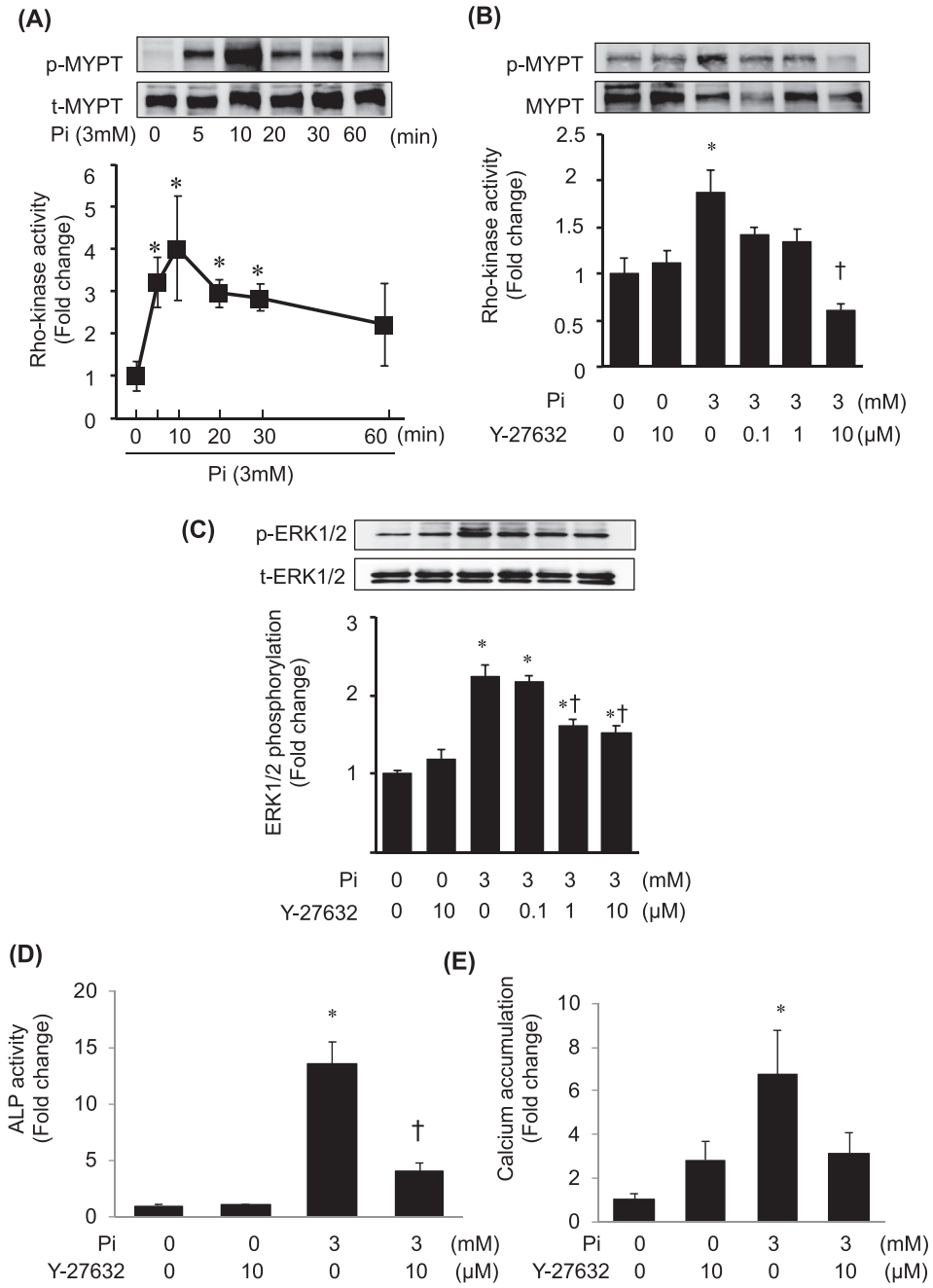


**Fig. 2.** Effect of Pi-induced ROCK activity on calcification signaling. (A) Time-course of Pi-induced MYPT phosphorylation in RASMCs. Cells were pretreated with the ROCK inhibitor Y-27632 for 30 min, then stimulated with Pi. MYPT phosphorylation (B) and ERK1/2 phosphorylation (C) were measured using western blotting. ALP activity (D) and calcium accumulation (E) were measured after 8 days' Pi stimulation with or without Y-27632. Values were analyzed using two-way ANOVA for repeated measures. *$P < 0.05$ vs. control, †$P < 0.05$ vs. Pi alone.

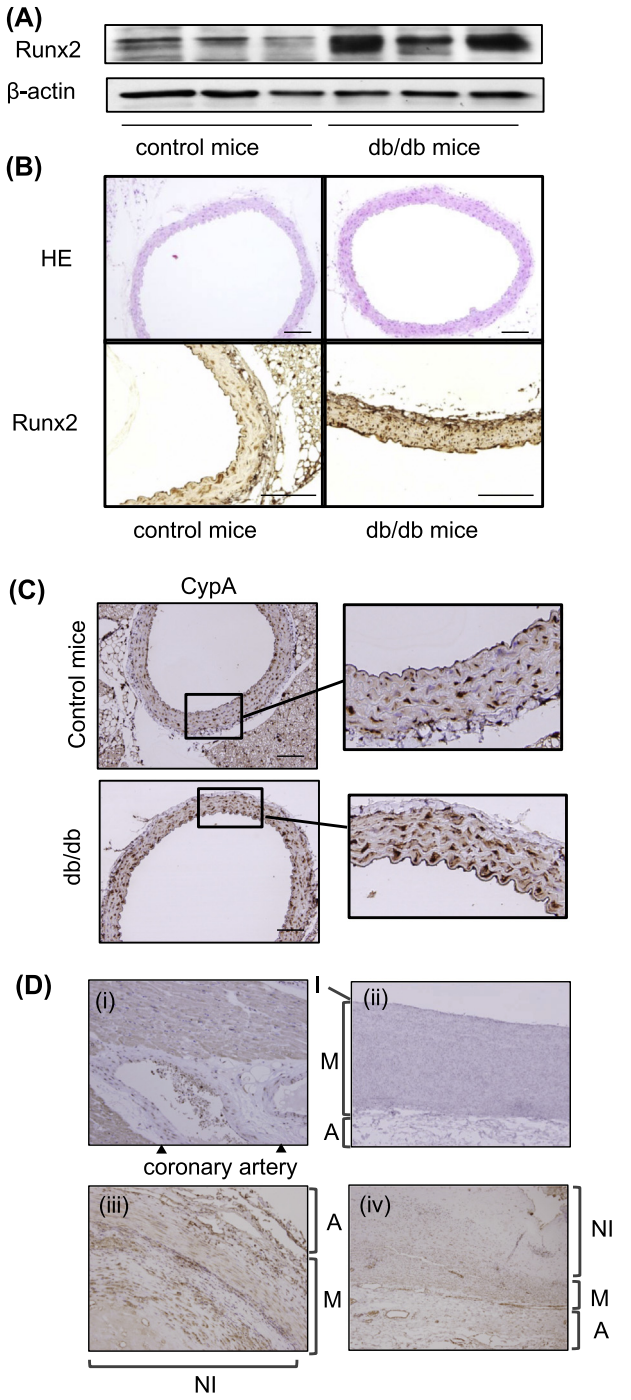


**Fig. 3.** Expressions of runx2 and CypA in aortas from diabetic mice or patients with atherosclerotic plaques. Runx2 expression in aortas from control or db/db mice was detected using western blotting (A) and immunohistochemistry (B). (C) CypA expression was analyzed in aortas from control or db/db mice. Right-hand panels show magnifications of left-hand panels. (D) CypA expressions in (i) myocardium, (ii) normal aorta, (iii) basilar artery with plaque, and (iv) aorta with plaque were detected using immunohistochemistry. Normal aorta and myocardium were sampled from healthy subjects. Atherosclerotic basilar artery and aorta with plaques were obtained from a diabetic patient. Each panel shows a representative picture. Scale bar: 0.1 mm.

and human arteries from autopsy samples similarly showed greater CypA expression in patients with diabetes mellitus (Fig. 3D). CypA was more abundant in myocardium and leukocytes compared to vascular wall cells in healthy subjects (Fig. 3Di, ii). However, increased CypA expression was observed in the basilar artery (Fig. 3Diii) and aorta with plaque (Fig. 3Div), but not in the normal coronary artery (Fig. 3Di) or aorta (Fig. 3Dii).

### 3.4. CypA is involved in Pi-induced calcification signaling in RASMCs

CypA secretion increased under 3 mM Pi stimulation with a peak 10 minutes after stimulation (Fig. 4A) but was inhibited by pretreatment with Y-27632 (Fig. 4B). The CypA inhibitor suppressed expression of Pi-induced ERK1/2 activation, runx2 expression, and calcium accumulation (Fig. 4C–E). Moreover, treatment with recombinant CypA increased calcium accumulation in RASMCs (Fig. 4F). These results suggest that autocrine CypA is mediated by ROCK activity and is involved in Pi-induced ERK1/2 phosphorylation and subsequent calcification in RASMCs.

## 4. Discussion

Consistent with previous reports,[3,5] our results indicate that the ROCK inhibitor Y-27632 inhibited the Pi-induced calcification signaling pathway, including CypA secretion (Fig. 4B), ERK1/2 phosphorylation (Fig. 2C), runx2 expression (Fig. 4D), ALP activity, and ultimately calcium accumulation (Fig. 2D, E) in RASMCs. This suggests that ROCK activity might trigger high phosphate-induced VSMC calcification. Therefore, ROCK inhibition may be an effective strategy for protecting against vascular calcification.

This study is the first to demonstrate the involvement of CypA in vascular calcification. CypA inhibition suppressed Pi-induced ERK1/2 phosphorylation and subsequent runx2 expression (Fig. 4C, D) in a manner similar to that of ROCK inhibition (Figs. 2C and 4D). Since the ROCK inhibitor Y-27632 suppressed CypA secretion in culture media under Pi stimulation (Fig. 4B), ROCK may be active upstream of autocrine CypA, after ERK1/2 phosphorylation. This is further supported by previous findings that CypA secretion requires ROCK activity-dependent vesicle formation in VSMCs.[20] Treatment of RASMCs with recombinant CypA peptide instead of Pi indicates that CypA alone can induce a comparable level of calcium accumulation (Fig. 4F). These *in vitro* findings strongly support the hypothesis that extracellular CypA may work as a mitogen to induce the calcification pathway in VSMCs.

Several clinical studies have previously demonstrated increased plasma levels of CypA in patients with type 2 diabetes mellitus, diabetic nephropathy, and carotid stenosis,[21–23] implying the possibility of CypA as a risk marker for cardiovascular events. Our findings demonstrate that CypA expression is upregulated in atherosclerotic or diabetic vasculature in both human patients and mice (Fig. 3). This is especially evident in the medial area of the aorta in diabetic db/db mice (Fig. 3C). A similar localization of runx2, a marker of osteoblast-like cell differentiation, could also be observed (Fig. 3A, B). These observations imply that increased CypA has a role in vascular calcification.

The role of the ROCK-CypA axis in Pi-induced VSMC calcification suggests that inhibitors of ROCK or CypA may be effective against VSMC calcification. However, some reports indicate an opposite relationship, wherein the rhoA-ROCK pathway negatively regulates cell calcification and osteoblast-like cell differentiation.[24,25] Chen et al. reported that the ROCK pathway increased the uptake of fetuin-A, an inhibitory factor of vascular calcification, working as a negative regulator.[24] Their observations showed opposite results to our present study. ROCK seems to have multifaceted roles in calcification signaling dependent of the calcification inducer, cell types, and so on. Interestingly, Gu et al. reported that ROCK activity is associated with increased nodule formation of valvular interstitial cells, but ROCK inhibition also increased ALP activity.[25] These

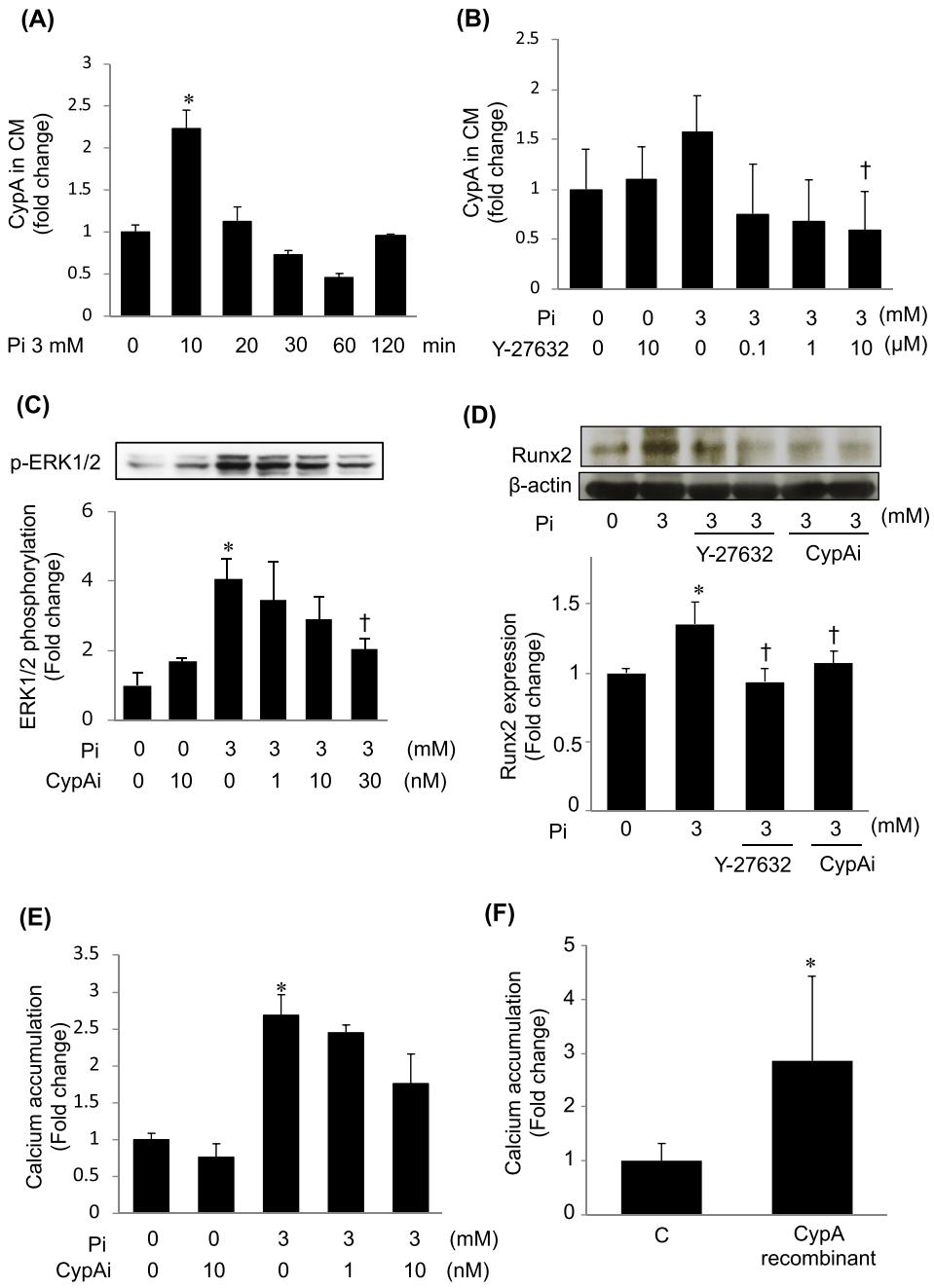


**Fig. 4.** Autocrine CypA mediates Pi-induced calcification signaling in RASMCs. Conditioned media (CM) after Pi stimulation were collected and condensed to measure CypA secreted from RASMCs. Panel (A) shows the time course and panel (B) shows the effect of the ROCK inhibitor Y-27632. The effects of 30 min' pretreatment by CypA inhibitor (CypAi) on ERK1/2 phosphorylation (C), runx2 expression (D), and calcium accumulation (E) were examined using western blotting and Calcium-E test Wako. Upper panels show representative blots and lower panels show the quantified intensity. (F) Human recombinant CypA (10 ng/mL) was added into culture media for 8 days, after which RASMCs were harvested and calcium accumulation was measured. Two-way ANOVA for (A) to (E) and Student's *t*-test for (F) were performed. $^{*}P < 0.05$ vs control, $^{\dagger}P < 0.05$ vs. Pi alone.

opposing roles of ROCK activity in vascular calcification and cell mineralization remain controversial.

One study suggests CypA may positively regulate cell mineralization, with CypA knockout (*Ppia*−/−) mice showing less skeletal volume compared to wildtype mice, and CypA overexpression leading to an increase in osteogenic markers such as ALP activity and runx2 expression.[14] However, cyclosporine A, a major inhibitor of CypA peptidyl-prolyl isomerase activity, aggravates VSMC calcification.[26] These findings contradict our observation that CypA inhibition suppressed Pi-induced ERK1/2 phosphorylation and runx2 expression (Fig. 4C, D). The CypA inhibitor 239836 which was used in this study shows 27 times more potent peptidyl-prolyl isomerase inhibitory activity than cyclosporine A, according to the commercial instruction of the reagent. The comparison of cyclosporine A and the CypA inhibitor 239836 will be needed to confirm the efficacy of CypA inhibition on cell calcification.

As demonstrated in the present study, vascular calcification is dependent on the same molecular pathways that are associated with intracellular signaling during bone mineralization *in vitro*, including ROCK, ERK1/2, and runx2 pathways. However, vascular

and other ectopic calcification occurs during bone loss or osteoporosis *in vivo*.[27] Therefore, further experiments should be carried out to clarify the pathophysiological roles of the ROCK-CypA axis in vascular calcification and the clinical effectiveness of ROCK or CypA inhibition on vascular calcification in a comprehensive manner.

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## Declaration of Competing Interest

The authors declare no conflicts of interest.

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