

Phosphodiesterase 3 inhibitors boost bone outgrowth
(ホスホジエステラーゼ 3 阻害薬は骨伸長を促進する)

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Abstract

Background and Purpose

C-type natriuretic peptide (CNP) stimulates skeletal growth by acting on the growth plates of long bones, and a CNP variant is clinically used for achondroplasia treatment. We previously reported that CNP stimulates the autonomic Ca^{2+} influx mediated by TRPM7 channels in growth plate chondrocytes to facilitate extracellular matrix synthesis for bone growth. In this study, we attempted to stimulate CNP signalling using phosphodiesterase (PDE) inhibitors.

Experimental Approach

Based on gene expression data, we focused on the role of PDE3B in growth plates. We performed imaging, biochemical and histological analyses in growth plate chondrocytes and ex vivo and in vivo analyses on bone and skeletal growth to address the pharmacological effects of PDE3 inhibitors.

Key Results

The representative PDE3 inhibitors cilostazol and milrinone elevated cGMP levels and activated cell-surface K^{+} channels probably due to protein kinase G-mediated phosphorylation in growth plate chondrocytes. The resulting hyperpolarization likely

facilitated TRPM7-mediated Ca^{2+} influx by increasing the Ca^{2+} -driving force. Moreover, cilostazol stimulated the elongation of cultured bones and enlarged the body size of juvenile mice.

Conclusion and Implications

Several PDE3 inhibitors have been used for clinical treatment of thrombosis, heart failure and asthma, while our observations suggest that the repositioning of PDE3 inhibitors would provide novel medications for skeletal diseases characterized by short stature.

Abbreviations

AST	aspartate aminotransferase
BK	big-conductance Ca^{2+} -dependent potassium
CaMKII	Ca^{2+} /calmodulin-dependent protein kinase II
CK	creatinine kinase
ECM	extracellular matrix
NPR2	natriuretic peptide receptor 2 (GC-B)
PLC	phospholipase C
TCA	trichloroacetic acid

Introduction

Skeletal long bones develop through endochondral ossification, during which primitive chondrocytes initially form growth plates at both ends of bone rudiments [1]. The growth plates then form three distinct zones, each of which contains chondrocytes that are homogenous in cell shape and maturation stage: ‘round chondrocytes’ differentiated from chondroblasts at most ends, ‘columnar chondrocytes’ characterized by a unique flat shape, and nonproliferating ‘hypertrophic chondrocytes’ undergoing apoptosis at the bone rudiment centre [2]. Bone growth mainly depends on the proliferation of round and columnar chondrocytes and characteristic extracellular matrix (ECM) synthesis, while genetic and environmental factors naturally affect cellular functions. In the hypertrophic chondrocyte zone, the region containing apoptotic bodies is gradually replaced by trabecular bones through the actions of osteoclasts and osteoblasts during juvenile development [1].

The peptide hormone C-type natriuretic peptide (CNP) acts on proliferating chondrocytes expressing the CNP-specific receptor guanylate cyclase NPR2 (GC-B) in an autocrine/paracrine manner and promotes long bone growth [3-7]. Accordingly, loss- and gain-of-function mutations in the NPR2 gene were reported to cause acromesomelic dysplasia and skeletal overgrowth disorder, respectively [8-10]. Furthermore, the CNP analogue vosoritide is used for the clinical treatment of achondroplasia, which is characterized by short limb bones and is mainly caused by gain-of-function mutations in the

fibroblast growth factor receptor 3 (FGFR3) gene [11, 12].

In the transient receptor potential channel superfamily, TRPM7 (melastatin subfamily member 7) forms a cation-permeable channel and participates in divergent cellular processes including cell growth and adhesion in various cell types [13]. We previously reported that TRPM7 channels generate autonomic Ca^{2+} influx in proliferating growth plate chondrocytes, leading to the activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), which stimulates ECM synthesis and thus facilitates bone outgrowth [14]. In addition, we revealed that the CNP signalling axis is essential for facilitating bone outgrowth [15]. CNP-induced NPR2 activation elevates the cellular cGMP content and activates cGMP-dependent protein kinase (PKG), leading to the phosphorylation and activation of big-conductance Ca^{2+} -dependent potassium (BK) channels in growth plate chondrocytes. The resulting BK channel activation induces hyperpolarization to facilitate TRPM7-mediated Ca^{2+} entry by enhancing the Ca^{2+} driving force and thus stimulating CaMKII. The defined cGMP-driven signalling cascade, the PKG-BK channel-TRPM7 channel-CaMKII axis, seems to pinpoint target proteins for developing new therapeutic treatments for skeletal growth disorders (Figure 16). In this context, we attempted to increase the steady-state cGMP concentration using phosphodiesterase (PDE) inhibitors to promote CNP signalling. In this report, we demonstrate that a class of PDE inhibitors activates the CNP signalling to promote spontaneous Ca^{2+} influx in growth plate chondrocytes and thus stimulates long bone growth.

Methods

Reagents, primers and mice

The commercial reagents used in this study are listed in Table 1. The synthetic primers used for RT-PCR analysis and mouse genotyping are listed in Table 2. C57BL/6 mice were largely used in this study, while we used Institute of Cancer Research (ICR) mice only for immunochemical CNP measurements. All mice were supplied by Japan SLC Inc (Hamamatsu, Japan). For in vivo assessment, we used only male mice to avoid the effects of the menstrual cycle. For the other assessments, we used both male and female mice. Chondrocyte-specific *Trpm7* knockout (*Trpm7^{fl/fl}*, *11EnhCre^{+/-}*) mice were generated and genotyped as described previously [15]. Achondroplasia model mice (*Fgfr3^{ach}* mice) were generated as described in a previous report [16]: The activated fibroblast growth factor receptor 3 (FGFR3) transgene is driven by the *Col2a1* promoter and enhancer sequences in *Fgfr3^{ach}* mice (FVB/N background). All experiments in this study were conducted with the approval of the Animal Research Committee according to the regulations on animal experimentation at Kyoto University (approval no. 17-7-5). Animal studies are reported in compliance with the ARRIVE guidelines [17] and with the recommendations made by the *British Journal of Pharmacology* [18].

Randomization and blinding

All animals were randomly assigned to each experimental group. Experiments were not done blindly because the housing cages had to be labelled for animal housing and management.

Gene expression analysis

For gene chip analysis, distal epiphyses enriched in round chondrocytes were separated from embryonic day 18.5 (E18.5) femoral bones under a stereomicroscope and subjected to total RNA preparation using the commercial reagent Isogen (Nippon Gene Co., Tokyo, Japan). The resulting total RNA was reverse-transcribed and analysed using the GeneChip Mouse Genome 430 2.0 (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. The obtained data have been deposited in the National Center for Biotechnology Information-Gene Expression Omnibus (NCBI-GEO) database under accession number GSE105256. For quantitative RT-PCR analysis, the total RNA was reverse-transcribed using a commercial kit (ReverTra ACE qPCR-RT kit). The resulting cDNAs were analysed by real-time PCR (LightCycler 480 II, Roche, Rotkreuz, Switzerland), and the cycle threshold was determined from the amplification curve as an index for relative mRNA content in each reaction.

Metatarsal organ culture

Metatarsal bone rudiments were cultured as previously described [19]. Briefly, the central metatarsal rudiments were dissected from E15.5 C57BL/6, *Trpm7^{fl/fl}*, *Il1EnhCre^{+/-}* and *Fgfr3^{ach}* mice and cultured in alpha-minimum essential medium (α MEM) supplemented with $5\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ ascorbic acid, 1-mM β -glycerophosphate pentahydrate, $100\text{ units}\cdot\text{ml}^{-1}$ penicillin, $100\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ streptomycin and 0.2 w/v% bovine serum albumin (fatty acid free). The explants were analysed under a photomicroscope (BZ-X710, Keyence, Osaka, Japan) for size measurements using Fiji/ImageJ software (US. NIH). For histological analysis, the cultured bones were fixed in 4 w/v% paraformaldehyde, embedded in Super cryoembedding medium (Section-lab, Yokohama, Japan), and frozen in liquid nitrogen. Serial cryosections ($\sim 6\text{ }\mu\text{m}$ in thickness) were prepared and treated with a commercial haematoxylin and eosin solution (Wako Pure Chemical, Osaka, Japan) or alcian blue solution (Merck, Burlington, MA, USA) for photomicroscopic observation and image analysis.

In vivo assessment of cilostazol-induced mouse growth

C57BL/6 mice (3 weeks old) were intraperitoneally (i.p.) injected daily for 4 weeks with $10\text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ of cilostazol suspended in olive oil (Wako Pure Chemical); control mice were injected with olive oil. The naso-anal length and body weight were weekly measured as previously reported [20]; the cranium was fixed and the mouse body was fully stretched

under isoflurane anaesthesia (3% in air) for the measurement.

After the test period, the heart rate and blood pressure of the mice were measured using a tail-cuff sphygmomanometer (BP-98AL, Softron, Tokyo, Japan). After the measurement of heart rate and blood pressure, mice were anaesthetised with 5% isoflurane for induction and maintained with 3% isoflurane. Blood was drawn from the inferior vena cava under anaesthesia, and the mice were then euthanised by cervical dislocation for tissue collection. Blood was centrifuged at $1500 \times g$ for 15 min to collect the serum. Serum biochemical data for Na^+ , K^+ , Cl^- , Ca^{2+} , aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatinine kinase (CK) were obtained by Oriental Yeast Co. (Nagahama, Japan). Finally, the femoral and tibial bones were isolated from the mice and fixed in 4 w/v% paraformaldehyde, demineralized in 18 w/v% EDTA solution (pH 7.2) for 2 weeks, embedded in Super cryoembedding medium (Section-lab), and frozen in liquid nitrogen. Serial cryosections ($\sim 8 \mu\text{m}$ in thickness) were prepared and treated with a commercial alcian blue solution (Merck). Microscopic images were quantitatively analysed using Fiji/ImageJ software. The average cell density and alcian blue positive area were calculated from three independent visual fields ($300 \times 600 \mu\text{m}$) in each growth plate preparation.

Bone slice preparations

Femoral bones isolated from E17.5 mice were immersed in a physiological salt solution

(PSS): (in mM) 150 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 5.6 glucose and 5 HEPES (pH 7.4).

Longitudinal bone slices (40–100 µm thickness) were prepared using a vibrating microslicer (DTK-1000N, Dosaka EM Co., Kyoto, Japan) as previously described [15] and subjected to cyclic nucleotide measurements, Ca²⁺ imaging, membrane potential monitoring and immunochemical analysis.

Quantification of cAMP and cGMP

Epiphyseal specimens enriched in proliferating chondrocytes were removed from the femoral bone slices under stereomicroscope and treated with or without PDE3 inhibitors (3-µM cilostazol or 10-µM milrinone for 30 min) in PSS at 37°C. For cGMP quantification, the specimens (eight to 10 slices) were homogenized in 5 w/v% trichloroacetic acid (TCA, 0.3 ml), and for cAMP quantification, the specimens (four to five slices) were homogenized in 5 w/v% TCA (0.6 ml), using a homogenizer (NS-310E, Microtec Nichion, Funabashi, Japan). After the lysates were centrifuged at 1500 × g for 10 min, the precipitates were recovered for protein quantification (#23225, Pierce BCA assay, Thermo Fisher Scientific, Waltham, MA, USA), and the supernatants were ether-extracted to remove TCA and then subjected to cAMP and cGMP quantification using commercial kits (ADI-900-066A cAMP ELISA kit and ADI-900-014 cGMP ELISA kit, Enzo Life Sciences, Farmingdale, NY, USA); the coefficient of variation (CV) values are available on the manufacture's webpage

(<https://www.enzo.com/product/direct-camp-elisa-kit/>,

<https://www.enzo.com/product/direct-cgmp-elisa-kit/>).

Quantification of CNP

Femoral bones were isolated from E17.5 ICR mice, promptly bisected and cultured in a physiological salt solution (PSS): (in mM) 150 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 5.6 glucose and 5 HEPES (pH 7.4). Conditioned solutions were corrected as indicated in Figure 1A, and loaded onto C-18 Sep-pak resin cartridges (Waters Associates, Milford, MA, USA) prewashed with 10% acetonitrile/0.1% trifluoroacetic acid. After extensive washing, the resins, peptide fractions were eluted with 50% acetonitrile/0.1% trifluoroacetic acid and concentrated by freeze drying. CNP in the peptide fractions were quantified by our sandwich chemiluminescent enzyme immunoassay (CLEIA) developed utilizing two monoclonal antibodies against the N-terminal region (capture antibody) and C-terminal ring portion (detection antibody). CNP is present as three molecular forms in various tissues, where CNP-53 comprises >50%, and both CNP-22 and proCNP account for ~20% [21]. Because the CNP CLIEA recognizes both CNP-53 and proCNP, more than 75% of total CNP in bio-samples are presumed to be quantified in our measurements. The CNP chemiluminescent enzyme immunoassay (CLEIA) detects CNP-53 down to 0.015 pM and measures it from 0.05 to 250 pM, and shows no cross-reactivity with atrial (A-type) natriuretic peptide (ANP),

brain (B-type) natriuretic peptide (BNP) and other related peptides; the mean intra and inter assay coefficient of variation (CV) values are 11.1% and 8.1%, respectively.

Ca²⁺ imaging

Fura-2 Ca²⁺ imaging of bone slices was performed as previously described [15, 22]. Briefly, bone slices placed on glass-bottom dishes (Matsunami, Osaka, Japan) were incubated in PSS supplemented with 15- μ M Fura-2AM for 1 h at 37°C. Fluorescence microscopy distinguished round, columnar, and hypertrophic chondrocytes with characteristic morphological features in the bone slices loaded with Fura-2. For ratiometric imaging, excitation light at 340 and 380 nm was alternately delivered, and emission light at >510 nm was detected by a cooled EM-CCD camera (Model C9100-13; Hamamatsu Photonics, Hamamatsu, Japan) mounted on an upright fluorescence microscope (DM6 FS, Leica, Wetzlar, Germany) using a 40 $\times$ water-immersion objective (HCX APO L, Leica). In typical measurements, ~30 round chondrocytes were randomly examined in each slice preparation to select the Ca²⁺-fluctuation-positive cells generating spontaneous events (>0.025 in Fura-2 ratio) using commercial software (Leica Application Suite X), and recording traces from the positive cells were then analysed using Fiji/ImageJ software for examining Ca²⁺ fluctuation amplitude and frequency. Imaging experiments were performed at room temperature (~25°C), and PSS was used as the normal bathing solution. For the

pretreatments of each compound, bone slices were immersed in PSS with the indicated compound for 30 min at 37°C after Fura-2 loading.

Membrane potential monitoring

Membrane potential recording was performed as previously described [15]. Briefly, bone slices placed on glass-bottom dishes were perfused with PSS containing 200-nM oxonol VI (AnaSpec, Fremont, CA, USA) for 45 min at room temperature (~25°C). During the recording, hyperpolarization results in extrusion of the dye from cells to subsequently decrease the cellular fluorescence intensity, while depolarization increases the fluorescence intensity. For preparation of the calibration plot showing the relationship between the fluorescence intensity and membrane potential, PSS solutions containing 20-, 40-, 60- or 100-mM KCl were used as bathing solutions. Fluorescence images with excitation at 559 nm and emission at >606 nm were captured at a sampling rate of ~10 s using a confocal microscope system (FV1000, Olympus, Tokyo, Japan). For the pretreatments with each compound, bone slices were immersed in PSS with the indicated compound for 30 min at 37°C before oxonol VI loading.

Immunochemical analysis

For immunohistochemical analysis, bone slices were pretreated with or without cilostazol

and fixed in 4 w/v% paraformaldehyde. After treatment with 1 w/v% hyaluronidase to increase immunodetection [23, 24] and blocking with fetal bovine serum-containing solution, the bone slices were incubated with primary and Alexa 488-conjugated secondary antibodies and observed with a confocal microscope (FV1000). For immunoblot analysis, bone slices were lysed in the buffer containing 4 w/v% sodium deoxycholate, 20-mM Tris-HCl (pH 8.8) and a phosphatase inhibitor cocktail (100-mM NaF, 10-mM Na₃PO₄, 1-mM Na₂VO₃ and 20-mM β -glycerophosphate). The resulting lysate proteins were electrophoresed on SDS-polyacrylamide gels and electroblotted onto nylon membranes for immunodetection using primary and HRP-conjugated secondary antibodies. The antigen proteins were visualized using a chemiluminescence reagent and image analyser (Amersham Imager 600, Cytiva, Tokyo, Japan). The immunoreactivities yielded were quantitatively analysed using Fiji/ImageJ software. The mean value of the vehicle control group was set as 100% in each blotting analysis. All the immuno-related procedures used comply with the recommendations made by the *BJP* [25].

Data and statistical analysis

This manuscript complies with *BJP*'s recommendations and requirements on experimental design and analysis [26]. Statistical analysis was performed only when the sample size was at least five animals per group. Data from groups of a smaller size ($n < 5$) were defined as

‘preliminary’ data. The group size in each protocol was not determined by statistical methods. Outliers were not excluded in data analysis. The data obtained are presented as the means $\pm$ SEMs, with *n* values indicating the number of examined mice and used as independent values. Student's *t* test and ANOVA were used for two-group and multiple group comparisons, respectively (Prism 7, GraphPad Software Inc.): $P < 0.05$ was considered to indicate statistical significance. Post hoc tests were performed only if *F* achieved $P < 0.05$, and there was no significant variance inhomogeneity.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org> and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/2024 [27-29].

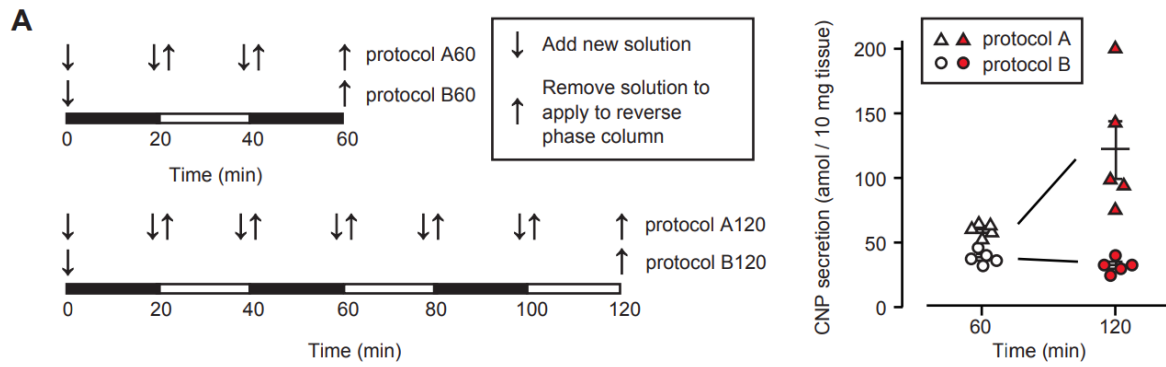
Results

CNP amount estimated in growth plate chondrocytes

Previous studies indicated that CNP is constitutively produced by growth plate chondrocytes and binds to activate NPR2 in an autocrine manner [30, 31]. In addition, the cartilage-specific *Nppc*-knockout results in severe short stature due to impaired long bone growth [31]. Therefore, autocrine CNP plays a critical role in bone elongation, although the concentration of CNP within growth plates has not been examined. To address this, we attempted to immunochemically quantify CNP leaked from embryonic femoral bones to extracellular solutions. Considering proteolytic inactivation of CNP, we designed two protocols to collect the conditioned solutions filling femoral specimens (Figure 1). In protocol A minimizing CNP inactivation, the conditioned solution was collected and refilled every 20 min. In protocol B mimicking physiological states, the conditioned solution was recovered only after incubation. Based on the CNP level detected in our enzyme immunoassay, steady-state extracellular CNP concentration was roughly estimated to be >12.4 pM in embryonic femoral growth plates. The reported EC₅₀ for NPR2 activation is 0.25 nM [32]. Our observation thus suggested that autocrine CNP partially and intermittently stimulates NPR2 to maintain cellular cGMP levels in growth plate chondrocytes.

PDE subtypes in growth plate chondrocytes

The aim of this study was to examine whether chemical PDE inhibitors can artificially stimulate bone growth by facilitating the CNP signalling in growth plate chondrocytes. Among the PDE family subtypes, 15 are presumed to physiologically contribute to cGMP inactivation in mammalian cell types [33-35]. Initially, we attempted to identify the PDE candidates that control the steady-state cGMP level in proliferating growth plate chondrocytes by microarray and RT-PCR analyses using RNA preparations from mouse embryonic femoral bones. Our gene expression data suggested that the cGMP-hydrolysing subtype genes *Pde1b*, *Pde2a*, *Pde3b*, *Pde5a*, *Pde6g*, *Pde9a* and *Pde10a* were moderately expressed in round chondrocytes (Figure 2). PDE1B is Ca^{2+} /calmodulin-dependently activated to hydrolyse both cAMP and cGMP [36], while PDE2A is activated by cGMP to balance cellular cAMP and cGMP levels [37]. Based on their biological features, PDE1B and PDE2A are unlikely to contribute to establishing the basal cGMP concentration under resting conditions. *Pde5a*-knockout mice exhibit small body sizes [38], and *Pde9a*-knockout mice



B V_{syn} : CNP synthesis rate (amol / hr / 10mg tissue)

k_{deg} : CNP degradation rate constant (1/hr)

$$\frac{d[\text{CNP}]}{dt} = V_{syn} - k_{deg}[\text{CNP}]$$

$[\text{CNP}]_A$: Collected CNP in protocol A60 (Hypothetical total CNP secretion)

$[\text{CNP}]_B$: Collected CNP in protocol B60 (Considering CNP degradation)

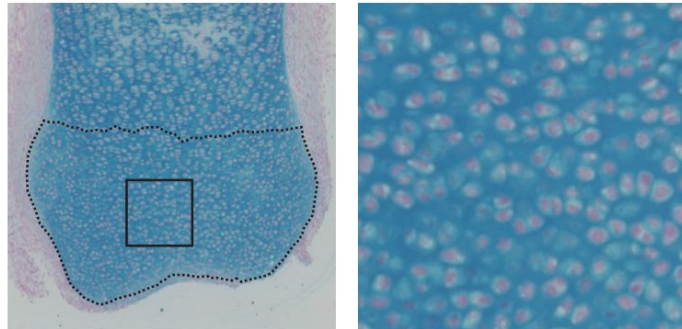
$$V_{syn} > [\text{CNP}]_{A60}$$

$$k_{deg} = \frac{V_{syn} - [\text{CNP}]_{B60}}{[\text{CNP}]_{B60} / 2} \quad (\text{assuming that physiological CNP inactivation is reflected in the protocol})$$

$$\text{At steady state} \quad \frac{d[\text{CNP}]}{dt} = 0 \quad \therefore [\text{CNP}] = \frac{V_{syn}}{k_{deg}}$$

C

Round area: $1.60 \pm 0.04 \times 10^5 \mu\text{m}^2$
 Extracellular region: $1.07 \pm 0.07 \times 10^5 \mu\text{m}^2$
 Extracellular region ratio: 66.7 %



D

CNP secretion (amol / 10 mg tissue)		k_{deg} (1/hr)	[CNP] in whole tissue (pM)	Estimated [CNP] in extracellular region (pM)
protocol A60	protocol B60			
61.3 ± 2.3	38.6 ± 2.4	0.74	8.3	>12.4

Figure1. Estimation of CNP concentration in growth plate cartilage.

(A) Two protocols for quantifying CNP leaked from growth plate cartilage are illustrated. In the left panel, upward arrows indicate the time points for adding new solutions, and downward arrows indicate the time points for removing conditioned solutions. The right graph summarizes the data obtained in CNP measurements; the data are presented as the mean $\pm$ SEM. (B) The formula for estimating a hypothetical steady-state concentration of CNP in growth plates. (C) Histological analysis of femoral bones from embryonic ICR mice. The femoral sections were stained with alcian blue (scale bar, 0.1 mm). The zonal size indicated by a dotted line and alcian blue-positive area were examined to determine the extracellular region ratio; the data are presented as the mean $\pm$ SEM. (D) Key parameters utilized for estimating extracellular CNP in growth plates.

have normal body sizes [39], suggesting that inhibitors of PDE5A and PDE9A are unlikely to stimulate bone growth. *Pde6g*-knockout mice develop retinitis pigmentosa blindness but have normal body sizes [40]. Human PDE6G and PDE10A mutations are responsible for blindness and parkinsonism, respectively, while no skeletal abnormalities have been found in either disease [41,42]. Therefore, PDE6G and PDE10A may not contribute to major bone growth signalling pathways. The processes of elimination highlighted PDE3B in the proliferating chondrocytes, and it is also notable that *Pde3b*-knockout mice exhibit the enlargement of the typical long bone tibia as a skeletal phenotype [43]. We thus focused on the pharmacological effects of clinically used PDE3 inhibitors on long bone development.

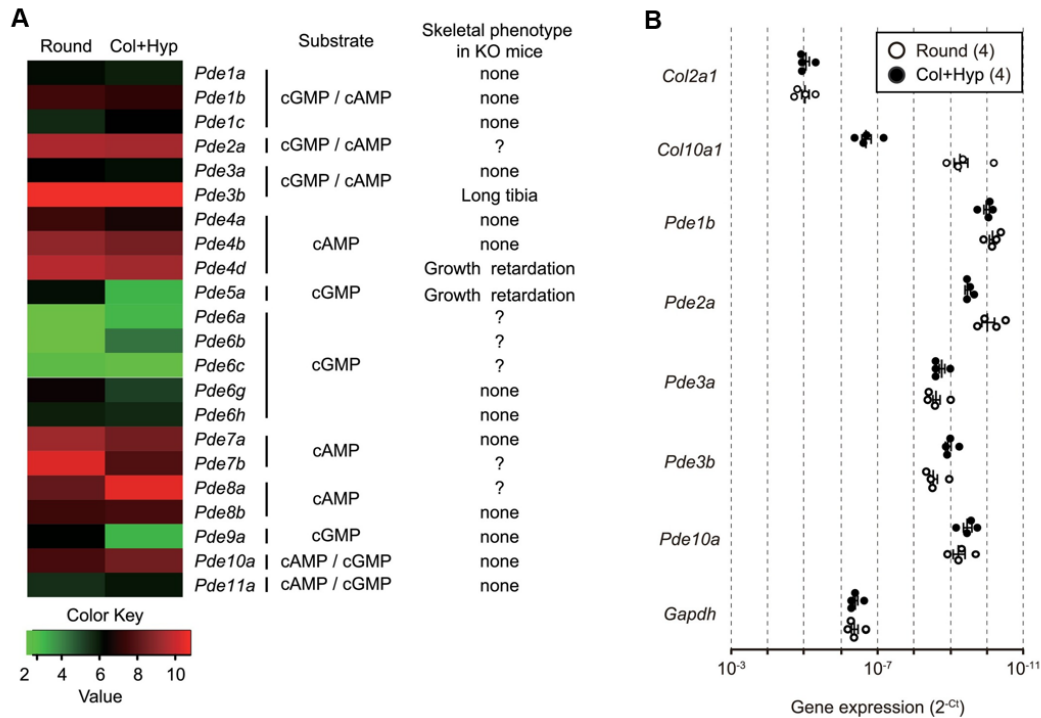


Figure2. Gene expression analysis in growth plate chondrocytes.

(A) Microarray heatmap representing the expression levels of PDE subtype genes in mouse growth plates. Growth plate sections enriched in either round chondrocytes (Round) or columnar and hypertrophic chondrocytes (Col + Hyp) were dissected from E18.5 femoral bones and subjected to total RNA preparation for microarray analysis. The resulting gene expression values are represented according to the colour scale. The substrate specificity and skeletal phenotype of each PDE subtype observed in knockout mice are also listed. (B) RT-PCR analysis of PDE subtype genes in growth plate sections in either round chondrocytes or columnar and hypertrophic chondrocytes. The primer sets used are listed in Table 2 and the cycle threshold (Ct) was determined for each RT-PCR amplification. The data are presented as the mean $\pm$ SEM, and the numbers of mice examined are shown in parentheses.

PDE3 inhibitors facilitate cultured bone growth

Ex vivo metatarsal culture has been exploited as a useful method for analysing endochondral bone growth [19]. We examined the effects of PDE inhibitors supplemented to culture medium on the longitudinal elongation of metatarsal bones prepared from embryonic mice. The representative PDE3 inhibitor cilostazol significantly stimulated bone outgrowth

during 4 days of culture (Figure 3A); the bone length increased by $120.7 \pm 0.9\%$ and $127.6 \pm 1.2\%$ in the control medium and 10- μ M cilostazol-containing medium, respectively. The other PDE3 inhibitors milrinone, anagrelide and olprinone also promoted cultured bone elongation, while the PDE2 inhibitor BAY-60-7550 and the PDE10 inhibitor MP10 had no effects (Figures 4 and 5A). Mutant mice carrying the chondrocyte-specific *Fgfr3* transgene have been used as an animal model of achondroplasia [44], and cilostazol supplementation was also effective in the mutant bones from the transgenic mice (Figure 6A). Therefore, PDE3 inhibitors seemed to commonly stimulate bone outgrowth in ex vivo culture.

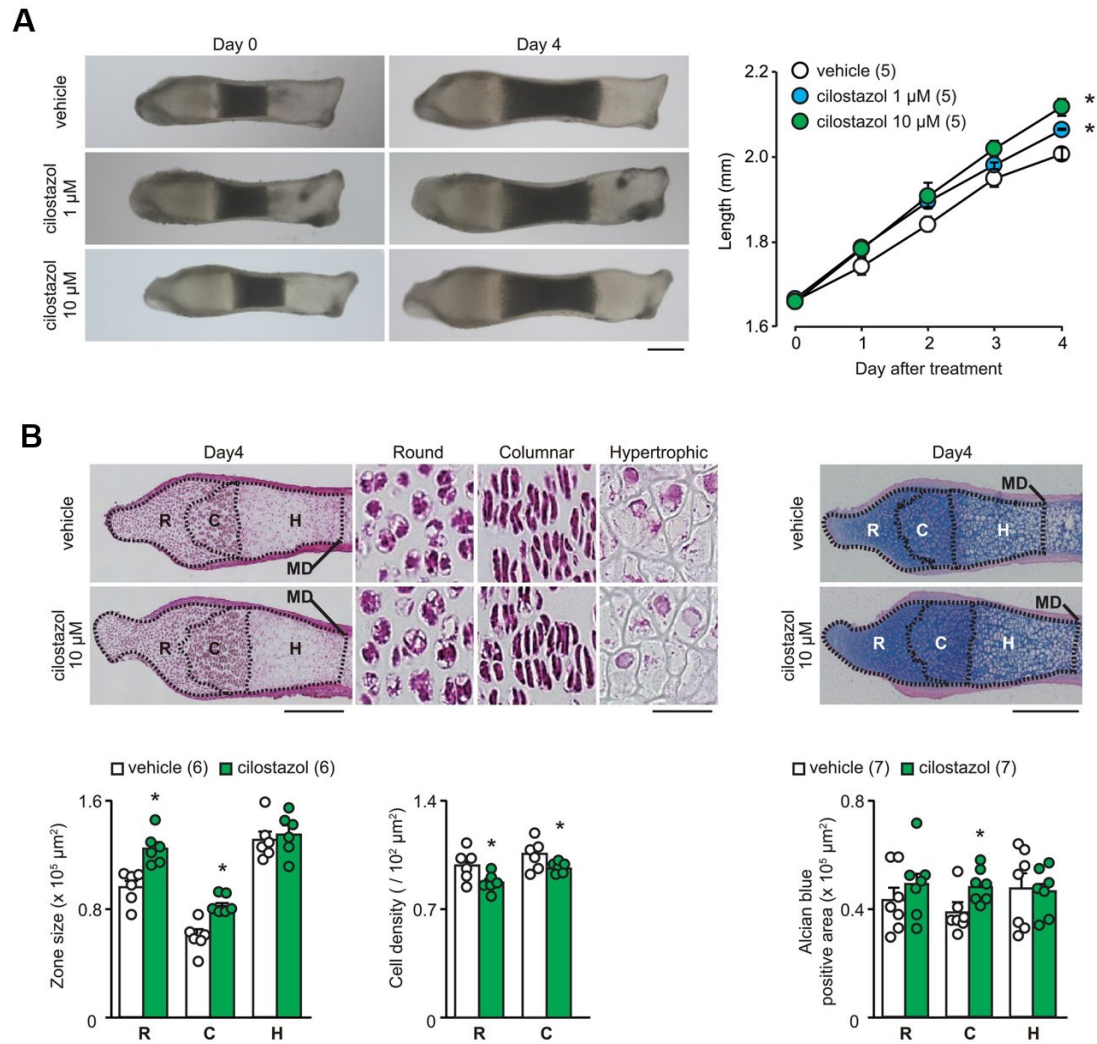


Figure3. Cilostazol promotes cultured bone growth.

Cilostazol-promoted outgrowth of cultured metatarsal bones. Representative images of the metatarsals before and after culture for 4 days in the presence or absence of cilostazol (left panels; scale bar, 0.3 mm). Longitudinal growth of the metatarsals was quantified over the culture period (right graph). The numbers of mice examined are shown in parentheses. The bone lengths at the endpoint were analysed and statistical differences from the vehicle-treated group are marked with asterisks (* $P < 0.05$ according to two-way ANOVA and Dunnett's test). (B) Histological analysis of metatarsal bones cultured in the presence or absence of cilostazol. The mid-longitudinal sections of cultured metatarsal bones were stained with haematoxylin and eosin (upper left panels; scale bar, 0.3 mm), and their high-magnification images of the round (R), columnar (C) and hypertrophic (H) chondrocyte zones are also shown (scale bar, 30 μ m). The metatarsal sections were likewise stained with alcian blue (upper right panels; scale bar, 0.3 mm). MD indicates the mid-diaphysis. In the round, columnar and hypertrophic chondrocyte zones, the zonal size, cell density and alcian blue-positive area were examined and are summarized (lower graphs). The data are presented as the mean $\pm$ SEM, and the numbers of mice examined are shown in parentheses. Statistical differences between the vehicle- and cilostazol-treated groups are marked with asterisks (* $P < 0.05$ according to t test).

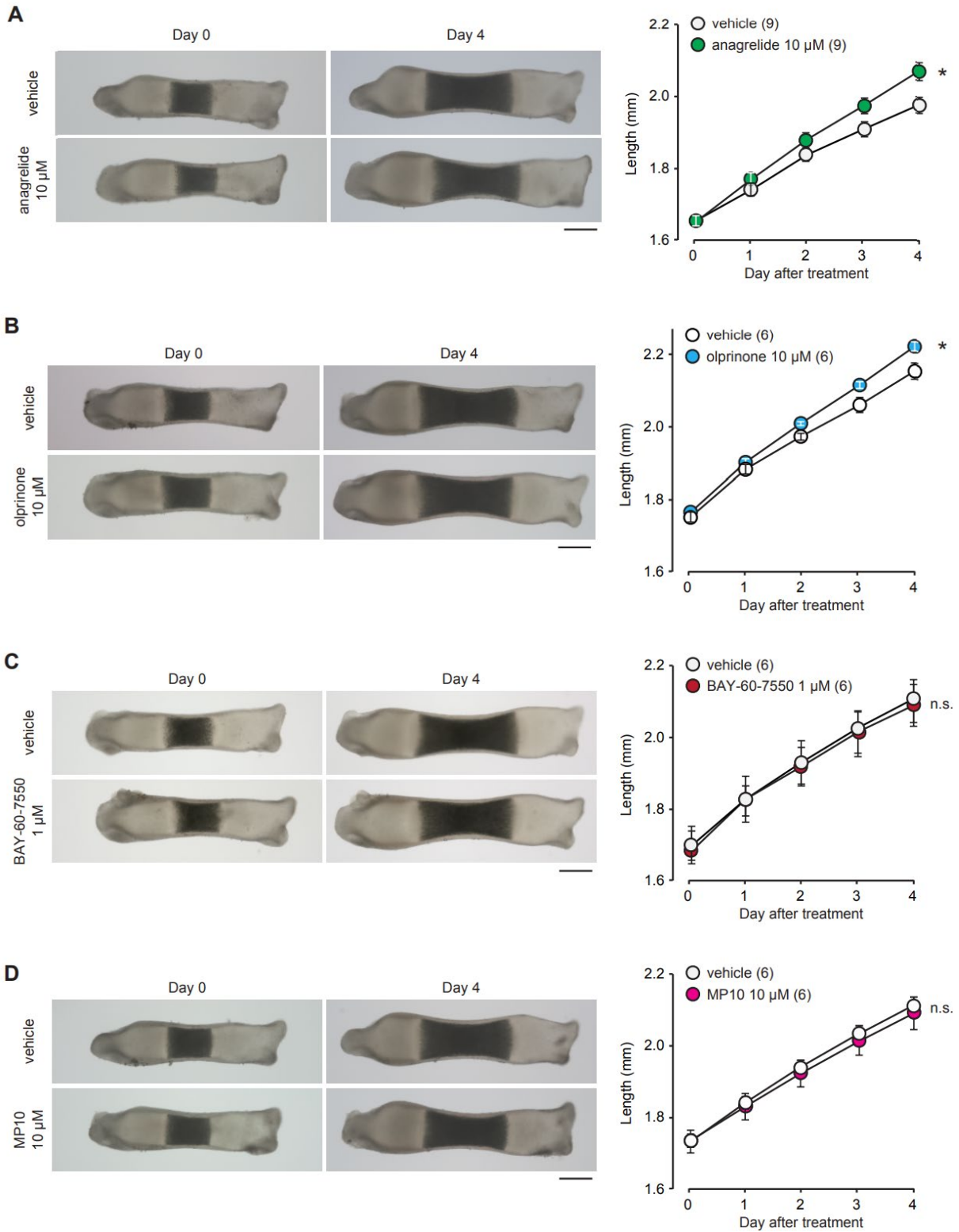


Figure4. Effects of various PDE inhibitors on cultured bone growth.

The effects of anagrelide (A), olprinone (B), BAY-60-7,550 (C) and MP 10 (D) on cultured metatarsal growth were examined. Representative images of the metatarsals before and after 4 days of culture in the presence or absence of the PDE inhibitors (left panels). Scale bar, 0.3 mm. Longitudinal bone growth cultured with or without the PDE inhibitors was quantified over the culture period (right graphs). The numbers of mice examined are shown in parentheses. The bone sizes were analysed in each experiment, and statistical differences at the endpoint from the vehicle-pretreated group are marked with asterisks ($*p < 0.05$ according to two-way analysis of variance [ANOVA] and Sidak's test).

Longitudinal sections were prepared from the cultured metatarsals and subjected to histological analysis of growth plates. In the cilostazol-treated bones, round and columnar chondrocyte zones were preferentially expanded (Figure 3B). Although the cell densities were slightly decreased, alcian blue-positive ECM regions tended to be enlarged in the extended proliferating chondrocyte zones. The milrinone-treated bones also exhibited histological features essentially the same as those of the cilostazol-treated bones (Figure 5B). Therefore, PDE3 inhibitors likely stimulated ECM production in proliferating chondrocytes to facilitate growth plate outgrowth towards bone elongation. Furthermore, the histological alterations induced by PDE3 inhibitors were essentially similar to those of CNP-treated cultured bones that we previously observed [15].

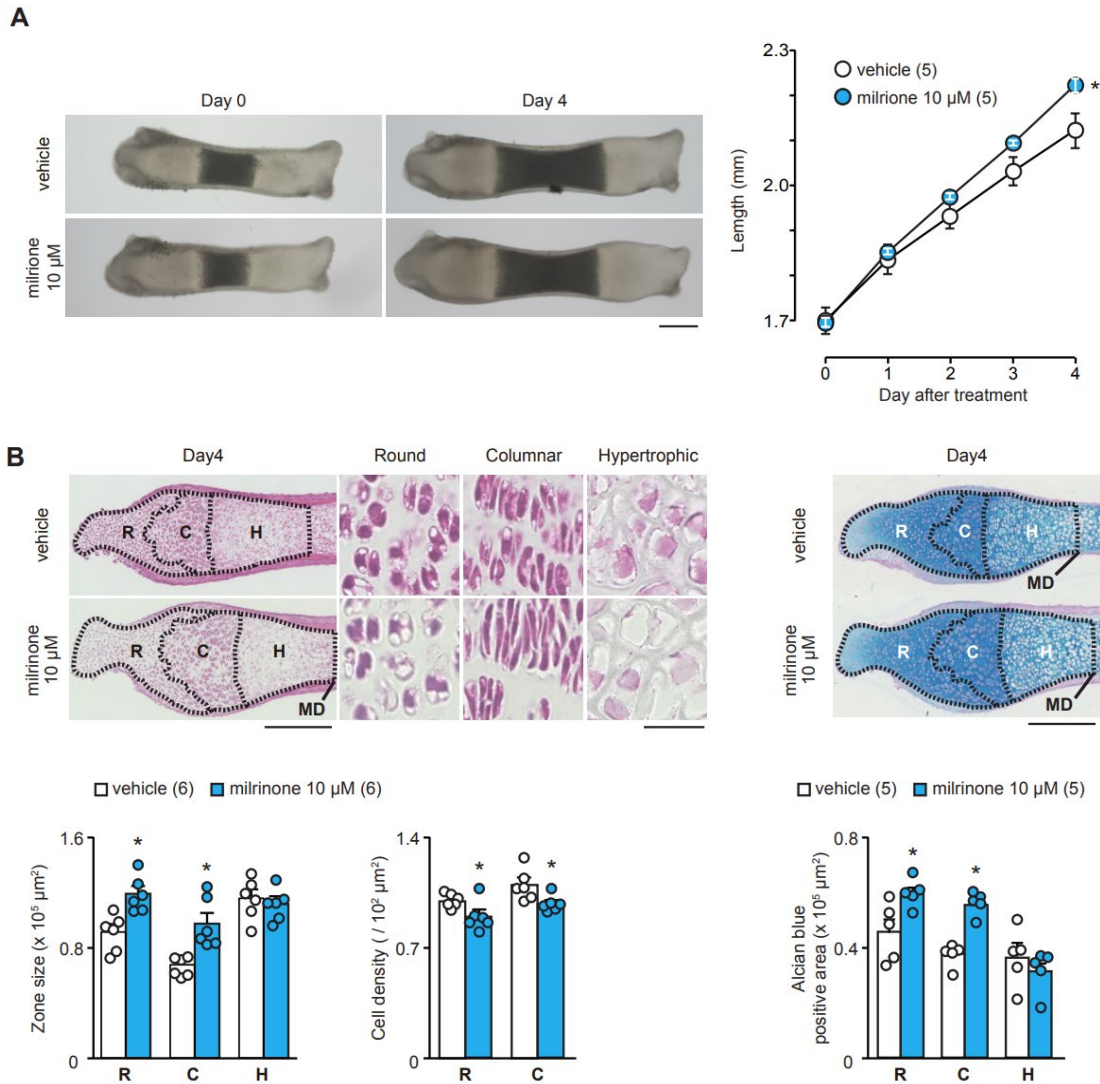


Figure5. Milrinone promoted cultured bone growth.

(A) Milrinone-promoted outgrowth of cultured metatarsal bones. Representative images of the metatarsals before and after culture for 4 days in the presence or absence of milrinone (left panels; scale bar, 0.3 mm). Longitudinal growth of the metatarsals was quantified over the culture period (right graph). The numbers of mice examined are shown in parentheses. The bone lengths were analysed at the endpoint, and statistical difference from the vehicle-treated group is marked with an asterisks (* $p < 0.05$ according to two-way ANOVA and Sidak's test). (B) Histological analysis of metatarsal bones cultured in the presence or absence of milrinone. The mid-longitudinal sections of cultured metatarsal bones were stained with haematoxylin and eosin (upper left panels; scale bar, 0.3 mm), and their high-magnification images from the round (R), columnar (C), and hypertrophic (H) chondrocyte zones are also shown (scale bar, 30 µm). The metatarsal sections were likewise stained with alcian blue (upper right panels; scale bar, 0.3 mm). MD indicates the mid-diaphysis. In the round, columnar and hypertrophic chondrocyte zones, the zonal size, cell density and alcian blue-positive area were examined and are summarized (lower graphs).

The data are presented as the mean $\pm$ SEM., and the numbers of mice examined are shown in parentheses. Statistical differences between the vehicle- and cilostazol-treated groups are marked with asterisks ($*p < 0.05$ according to *t* test).

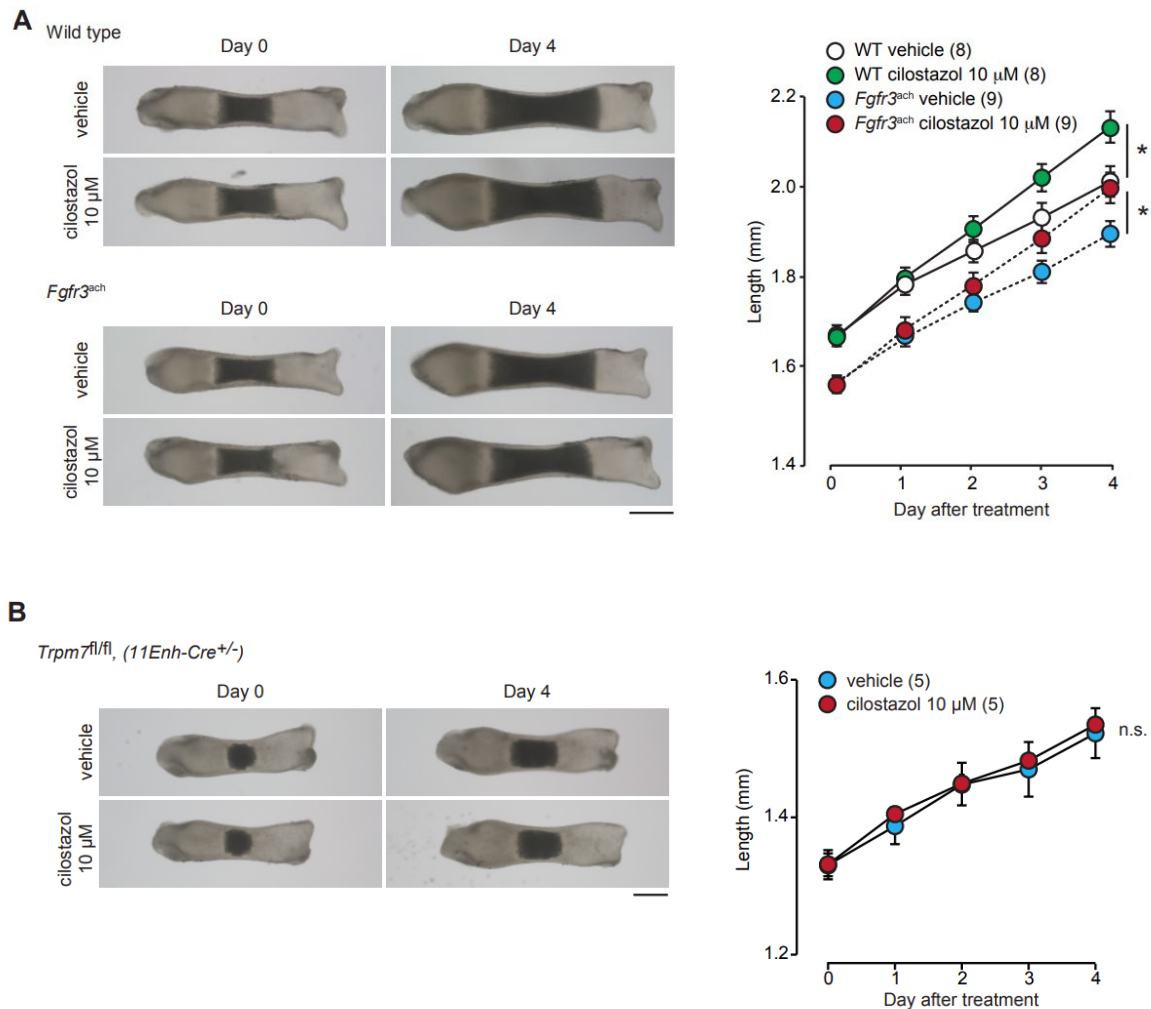


Figure 6. Effects of cilostazol on the mutant bone growth.

(A) Effects of cilostazol on the outgrowth of cultured metatarsals from achondroplasia model mice. Metatarsals isolated from control (wild-type) and achondroplasia model embryos (*Fgfr3^{ach}*) were precultured in regular medium for 5 days, and then test-cultured in medium supplemented with or without cilostazol for 4 days. Representative images of cultured metatarsals are shown (left panels; scale bar, 0.3 mm). Longitudinal bone outgrowth during the test period was statistically analysed in each genotype group (right graph). The numbers of mice examined are shown in parentheses. The bone sizes were analysed over the test period and statistical differences between the cilostazol-treated and nontreated groups for each genotype are marked with asterisks ($*p < 0.05$ according to two-way ANOVA and Sidak's test). (B) Cilostazol had no effect on the outgrowth of *Trpm7*-deficient bones.

Metatarsals isolated from chondrocyte-specific *Trpm7*-knockout embryos were precultured in regular medium for 5 days, and then test-cultured in medium supplemented with or without cilostazol for 4 days. Representative images of the metatarsals before and after the test are shown (left panels; scale bar, 0.3 mm), and the longitudinal growth of the metatarsals was examined over the test culture period (right graph). The numbers of mice examined are shown in parentheses, and the data obtained were statistically analysed (two-way ANOVA and Sidak's test; n.s., not significant).

Cilostazol stimulates mouse body growth

To examine the *in vivo* effects of PDE3 inhibitors on body growth, juvenile mice immediately after weaning (3 weeks old) were daily injected with cilostazol at 10 mg·kg⁻¹ (Figure 7A). During the 4-week test period, body size (naso-anal length) increased to 93.6 ± 0.3 mm in the vehicle-treated control mice and increased to 95.3 ± 0.5 mm in the cilostazol-treated mice. Therefore, cilostazol application potentiated body growth in young mice. After the test period, tibial sections were prepared from the mice and subjected to histological analysis (Figure 7B). As with the cultured metatarsal bones, the alcian-blue-positive ECM regions were expanded in the enlarged growth plates prepared from the cilostazol-treated mice (Figure 7C). Although cilostazol frequently causes hypotension and tachycardia as its side effects, the cilostazol treatment seemed to exert no influence on the major cardiovascular or haematological parameters during the test period (Figure 8). Based on the observations that PDE3 inhibitors stimulated cultured bone growth and juvenile mouse growth, we next focused on the pharmacological effects of cilostazol on growth plate chondrocytes.

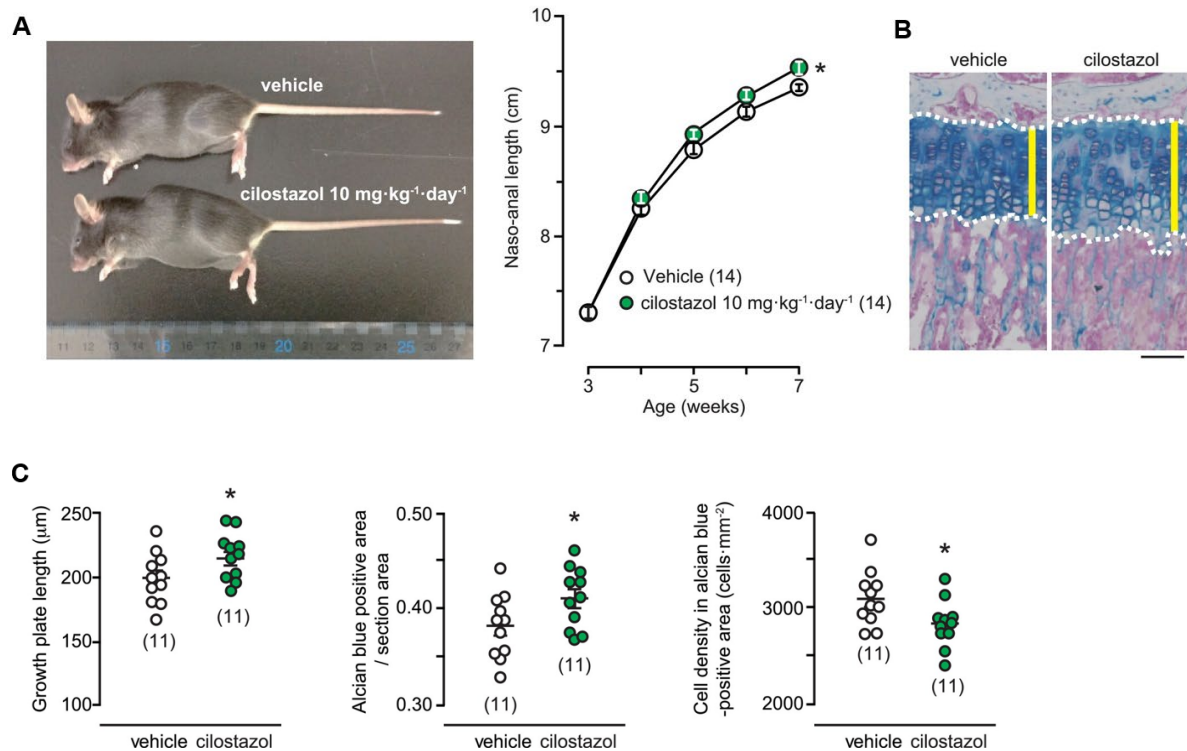


Figure 7. Cilostazol stimulates body growth in juvenile mice.

(A) Increased body length by daily intraperitoneal injection of cilostazol. Representative images of mice treated with vehicle or cilostazol for 4 weeks (left panel). Transitions in naso-anal length were analysed over the test period and the data are summarized (right graph). The numbers of mice examined are shown in parentheses, and statistical differences at the endpoint are marked with asterisks (* $P < 0.05$ according to two-way ANOVA and Sidak's test). (B) Representative images of alcian blue-stained tibial growth plates from the vehicle- and cilostazol-treated mice. The mice were drug-treated for 2 weeks (3- to 5-week-old) and then subjected to the histological analysis. Yellow bars indicate alcian blue-positive growth plate zone. Scale bar, 0.1 mm. (C) Tibial growth plate length, alcian blue-positive area per section area, and cell density in the alcian blue-positive area are summarized. The data are presented as the mean $\pm$ SEM, and the numbers of mice examined are shown in parentheses. Significant differences between the groups are indicated by asterisks (* $P < 0.05$ according to t test).

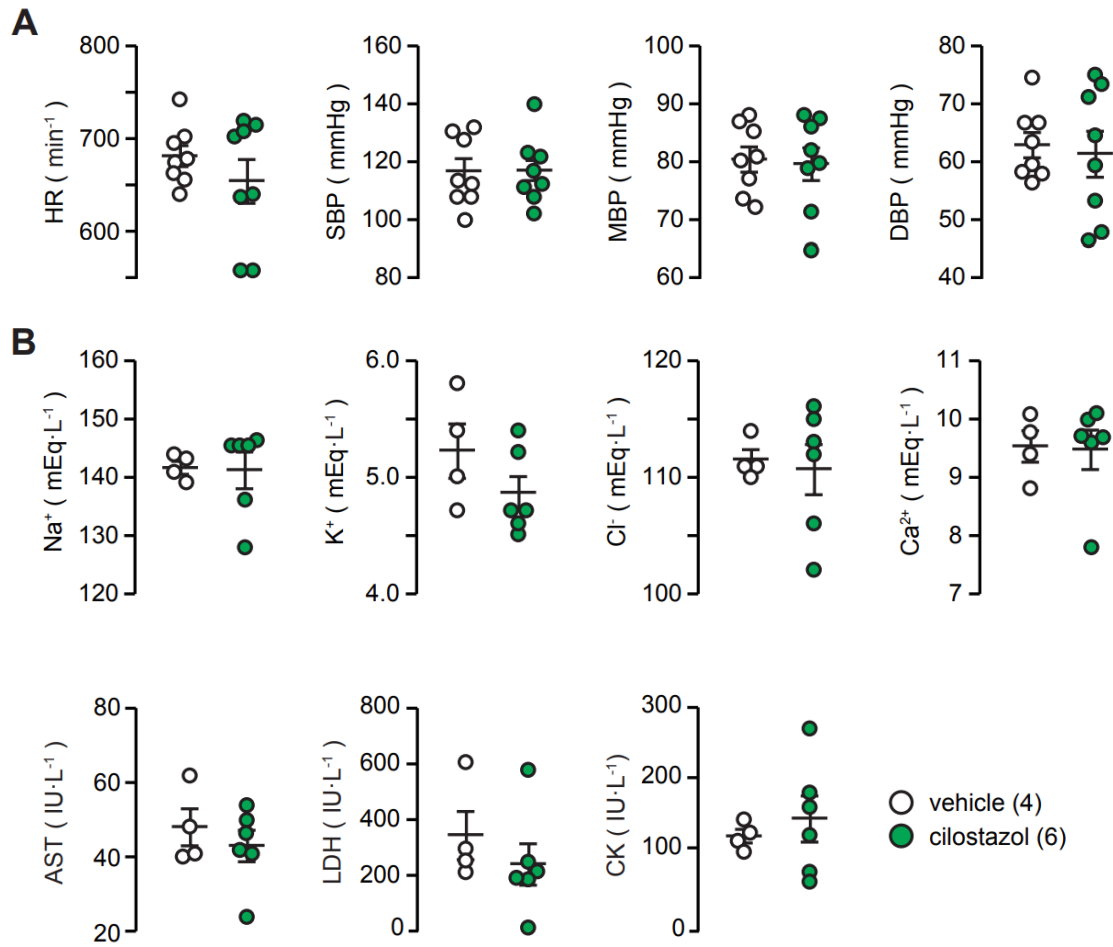


Figure 8. No obvious effects of in vivo cilostazol treatments on major cardiovascular and serological parameters.

After daily injection of cilostazol for 4 weeks, 7-week-old mice were examined. (A) Heart rate and blood pressure were measured by the tail-cuff method. (B) Mouse serum was prepared from blood drawn from the inferior vena cava and subjected to outsourced serological analyses. The data are presented as the mean $\pm$ SEM., and the numbers of mice examined are shown in parentheses.

Cilostazol elevates cGMP levels in growth plate chondrocytes

PDE3B hydrolyses both cAMP and cGMP, but its cAMP hydrolytic activity is attenuated by elevated cGMP content [34]. To determine the fundamental role of PDE3B in growth plate chondrocytes, we isolated femoral bones from embryonic mice and prepared the epiphyseal slices enriched in proliferating chondrocytes for the immunochemical quantification of

cAMP and cGMP levels. In response to cilostazol treatments to the slice specimens (3 μ M for 30 min), the cGMP content increased $\sim$ 1.7-fold, while the cAMP content remained unchanged (Figure 9A). Similar effects on the cyclic nucleotide content were also observed in the milrinone treatment group (Figure 10A). Therefore, PDE3 inhibitors seemed to selectively elevate cGMP concentration in growth plate chondrocytes.

Cilostazol stimulates Ca^{2+} entry in growth plate chondrocytes

In proliferating growth plate chondrocytes, TRPM7 channels spontaneously and intermittently open to generate intracellular Ca^{2+} fluctuations, and the TRPM7-mediated Ca^{2+} influx is facilitated by BK channel-induced hyperpolarization [14]. We next analysed the effects of cilostazol pretreatment (1 or 3 μ M for 30 min) on the Ca^{2+} fluctuations in round chondrocytes. In Fura-2 Ca^{2+} imaging of femoral bone slices, we observed that the cilostazol treatments remarkably facilitated the Ca^{2+} fluctuations; the fluctuation-positive cell rate, fluctuation amplitude and frequency were dose-dependently increased in the cilostazol-treated chondrocytes (Figure 9B). Furthermore, milrinone exerted effects on the Ca^{2+} fluctuations essentially similar to those of cilostazol (Figure 10B). Therefore, PDE3 inhibitors seemed to commonly facilitate Ca^{2+} fluctuations in proliferating chondrocytes.

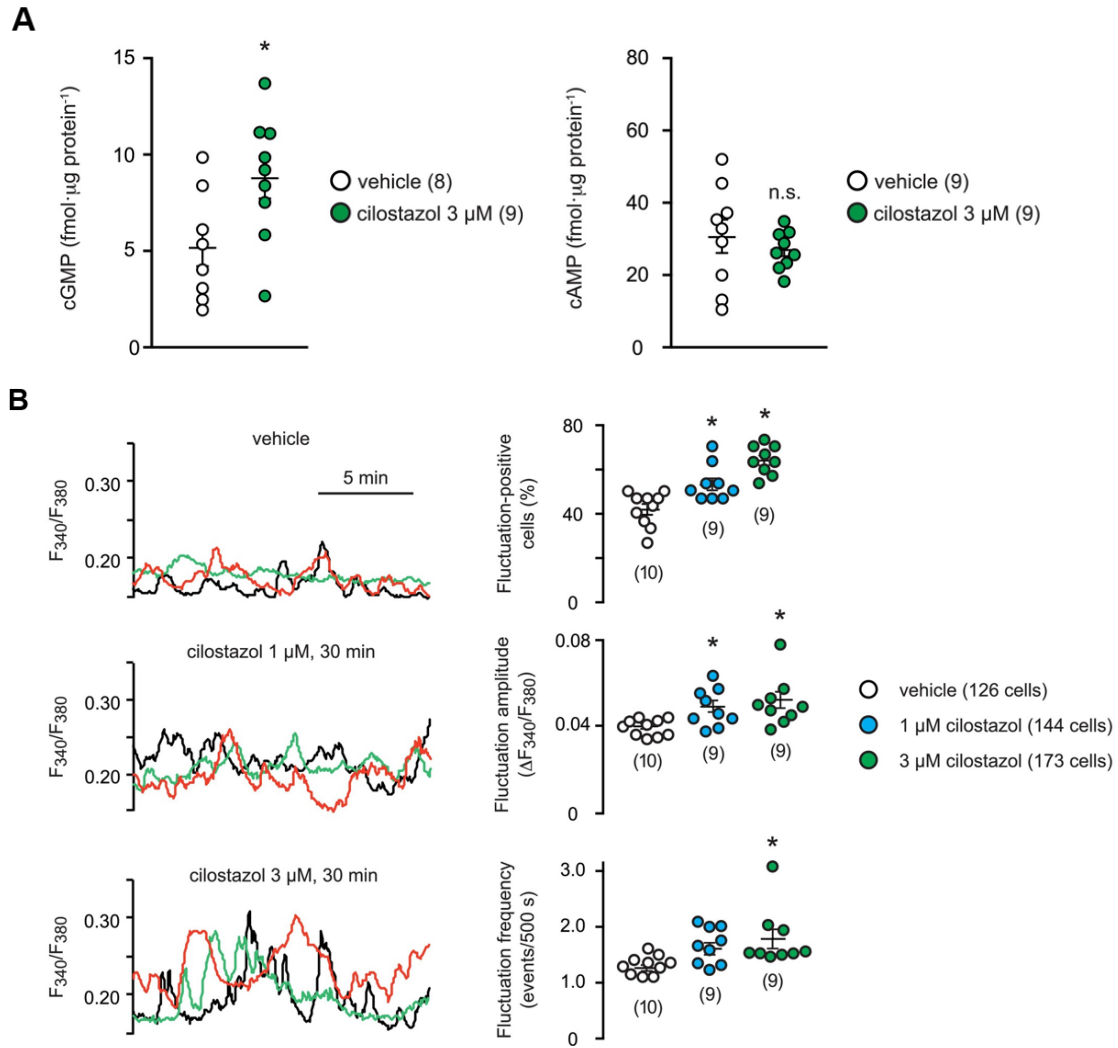


Figure 9. Cilostazol facilitates Ca²⁺ fluctuations in growth plate chondrocytes.

(A) Effects of cilostazol on cGMP and cAMP levels in growth plate chondrocytes. Femoral bone slices enriched in growth plate chondrocytes were treated with or without cilostazol for 30 min and then subjected to cyclic nucleotide quantification. The data are presented as the mean ± SEM, and the numbers of mice examined are shown in parentheses. A significant difference between the groups is indicated by an asterisk (**P* < 0.05, *t* test; n.s., not significant). (B) Effects of cilostazol on Ca²⁺ fluctuations in growth plate chondrocytes. Fura-2 imaging of round chondrocytes in femoral bone slices pretreated with or without cilostazol for 30 min. Representative recording traces from three cells are shown for each pretreatment group (left panels). The effects of cilostazol on spontaneous Ca²⁺ fluctuations are summarized (right graphs); the fluctuation-positive cell ratio, fluctuation amplitude and fluctuation frequency were statistically analysed. The numbers of mice and cells examined are shown in the bar graphs and keys, respectively. Statistical differences from the vehicle-pretreated group are marked with asterisks (**P* < 0.05, one-way ANOVA and Dunnett's test).

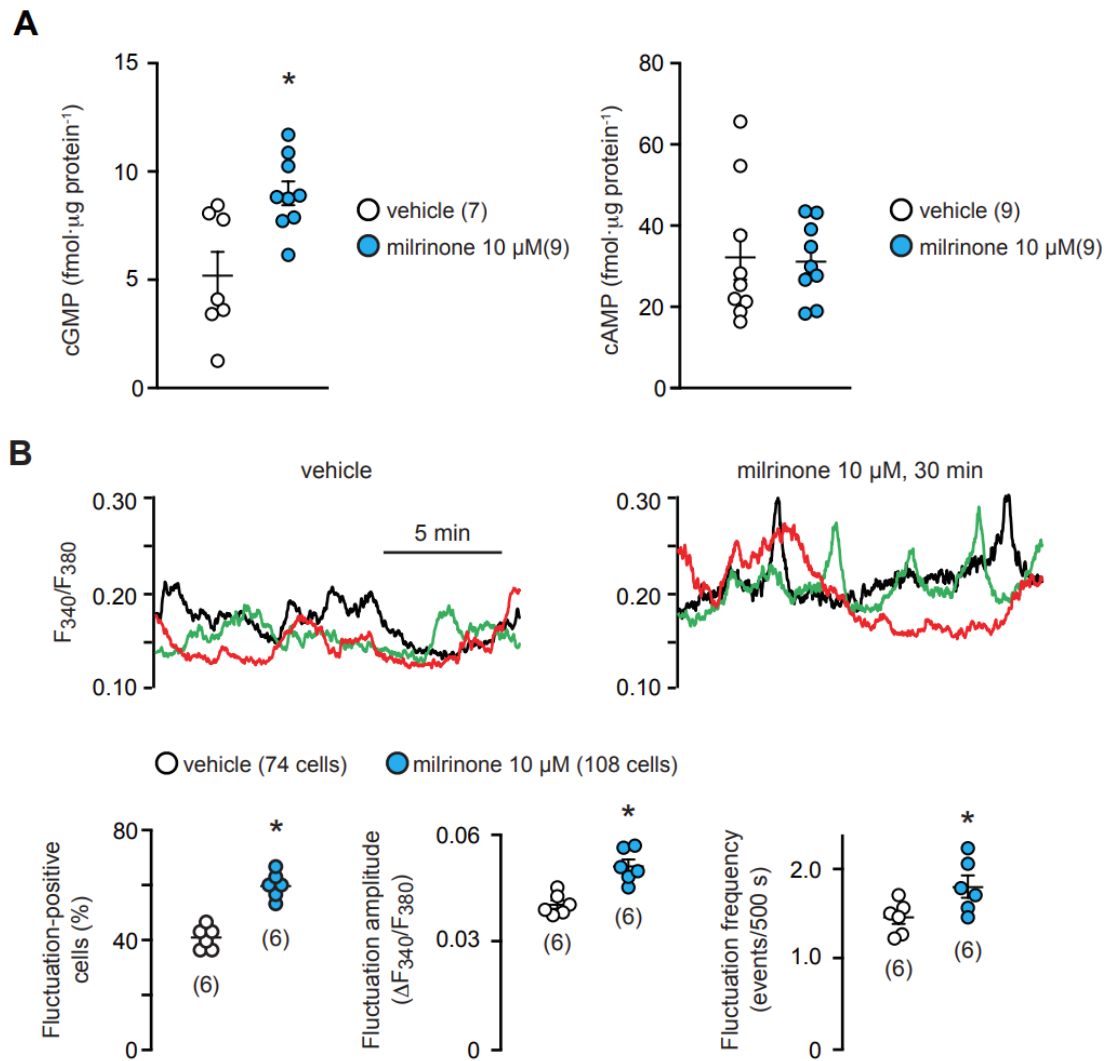


Figure 10. Milrinone facilitates Ca²⁺ fluctuations in growth plate chondrocytes.

(A) Effects of milrinone on cGMP and cAMP levels in growth plate chondrocytes. Femoral bone slices enriched in growth plate chondrocytes were treated with or without milrinone for 30 min and then subjected to cyclic nucleotide quantification. The data are presented as the mean ± SEM., and the numbers of mice examined are shown in parentheses. A significant difference between the groups is indicated by asterisks (**p* < 0.05 according to t test). (B) Effects of milrinone on Ca²⁺ fluctuations in growth plate chondrocytes. Fura-2 imaging of round chondrocytes in femoral bone slices pretreated with or without cilostazol for 30 min. Representative recording traces from three cells are shown for each pretreatment group (upper panels). The effects of milrinone on spontaneous Ca²⁺ fluctuations are summarized (lower graphs); the fluctuation-positive cell ratio, fluctuation amplitude and fluctuation frequency were statistically analysed. The numbers of mice and cells examined are shown in the bar graphs and keys, respectively. Statistical differences from the vehicle-pretreated group are marked with asterisks (**p* < 0.05 according to t test).

The PKG inhibitor KT5823 and the BK channel inhibitor paxilline clearly attenuated the cilostazol-facilitated Ca^{2+} fluctuations (Figure 11A, B), suggesting the involvement of the PKG and BK channels in the cilostazol-evoked signalling pathway. The TRPM7 inhibitor FTY720 almost abolished the facilitated Ca^{2+} fluctuations (Figure 11C), indicating that TRPM7-mediated Ca^{2+} entry was facilitated by cilostazol. Based on these observations, together with the cyclic nucleotide quantification data, we can reasonably propose that PDE3 inhibitors increased the cGMP content and stimulated PKG to induce BK channel phosphorylation, leading to the facilitation of TRPM7-mediated Ca^{2+} influx by inducing hyperpolarization in proliferating chondrocytes.

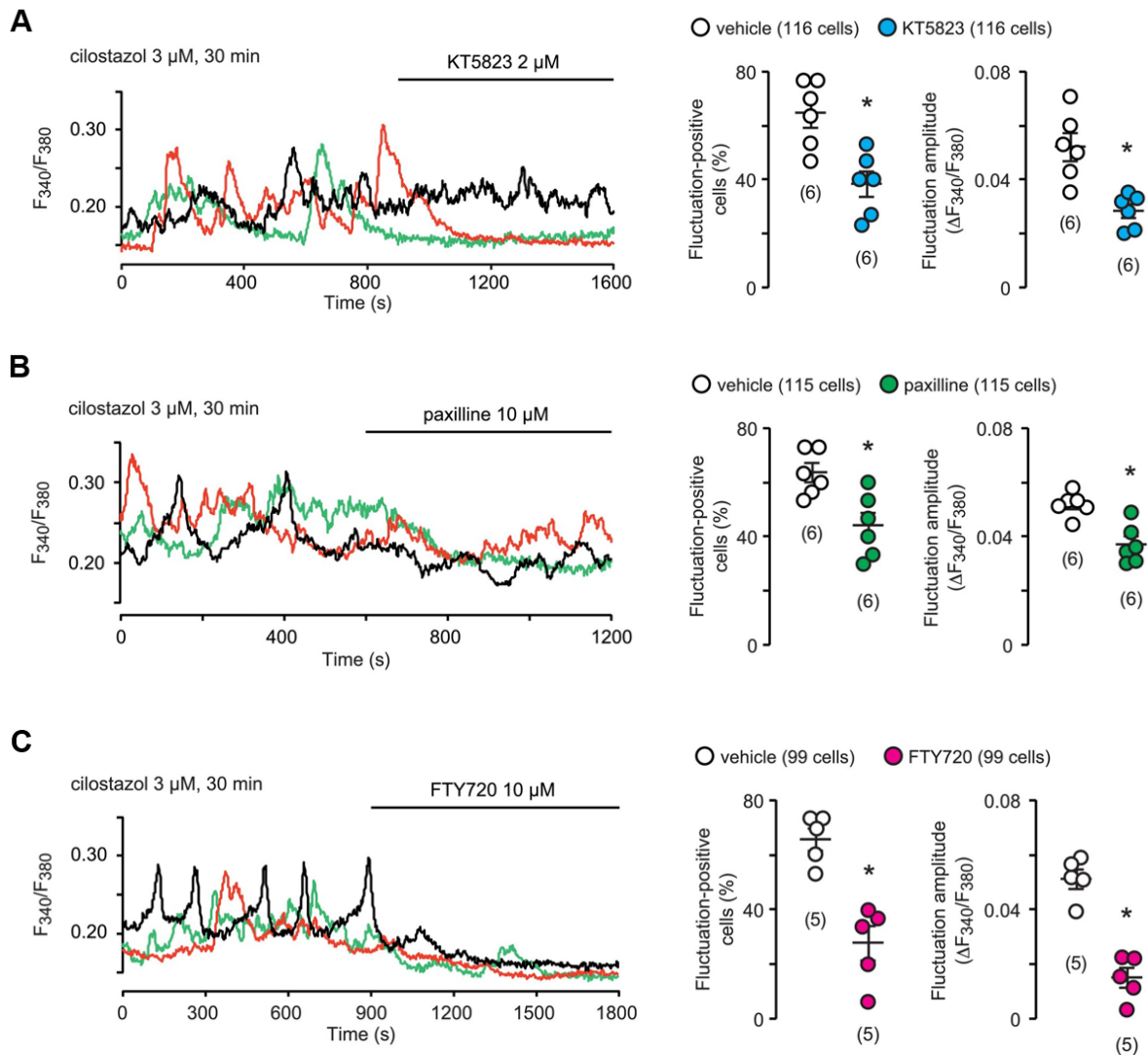


Figure 11. Pharmacological characterization of cilostazol-facilitated Ca^{2+} fluctuations.

(A) Effects of the PKG inhibitor KT5823 on cilostazol-facilitated Ca^{2+} fluctuations. Femoral bone slices were pretreated with cilostazol for 30 min and then subjected to Ca^{2+} imaging. Representative recording traces from three round chondrocytes are shown (left panel), and KT5823-evoked effects on Ca^{2+} fluctuations were summarized (right graphs). (B) Effects of the BK channel inhibitor paxilline on cilostazol-facilitated Ca^{2+} fluctuations. Representative recording traces (left panel) and a summary of paxilline-evoked effects are shown (right graphs). (C) Effects of the TRPM7 inhibitor FTY720 on cilostazol-facilitated Ca^{2+} fluctuations. Representative recording traces (left panel) and a summary of FTY720-evoked effects (right graphs) are shown. The data are presented as the mean $\pm$ SEM, and the numbers of mice and cells examined are shown in the bar graph and keys, respectively. Significant inhibitor-induced shifts are marked with asterisks ($*P < 0.05$ in t test).

Cilostazol reduces membrane potential in growth plate chondrocytes

Next, growth plate slices were subjected to confocal imaging using the voltage-dependent dye oxonol VI [45]. In this measurement, depolarization results in the accumulation of the dye in cells and thus linearly correlates well with the cellular fractional fluorescence intensity which is normalized to the maximum intensity monitored using a 100-mM KCl-containing bath solution. Under resting conditions, cilostazol pretreatment (3 μ M for 30 min) reduced the fractional intensities in growth plate chondrocytes (Figure 12A); the estimated resting potentials were -40.0 ± 0.2 and -41.2 ± 0.1 mV in the vehicle- and cilostazol-pretreated round chondrocytes, respectively. However, the addition of paxilline to a perfusion solution quickly elevated the fractional intensities and abolished the difference between the cilostazol- and vehicle-pretreated cells (Figure 12B). In the presence of the BK channel activator NS1619, the cilostazol- and vehicle-pretreated cells exhibited similar fractional intensities. Therefore, cilostazol likely activated BK channels to induce hyperpolarization.

In the CNP-evoked signalling cascade, PKG phosphorylates and activates BK channels (Figure 16). Even in the vehicle-pretreated control chondrocytes, pretreatment with KT5823 clearly elevated the resting membrane potential, suggesting that the mechanism of PKG-mediated BK channel activation was functioning under basal conditions (Figure 12C). Furthermore, the shift induced by KT5823 cotreatment was more obvious in the

cilostazol-pretreated cells, and KT5823 seemed to abolish the difference in membrane potential between the vehicle- and cilostazol-pretreated cells. Therefore, cilostazol likely enforced the PKG-mediated BK channel activation in growth plate chondrocytes.

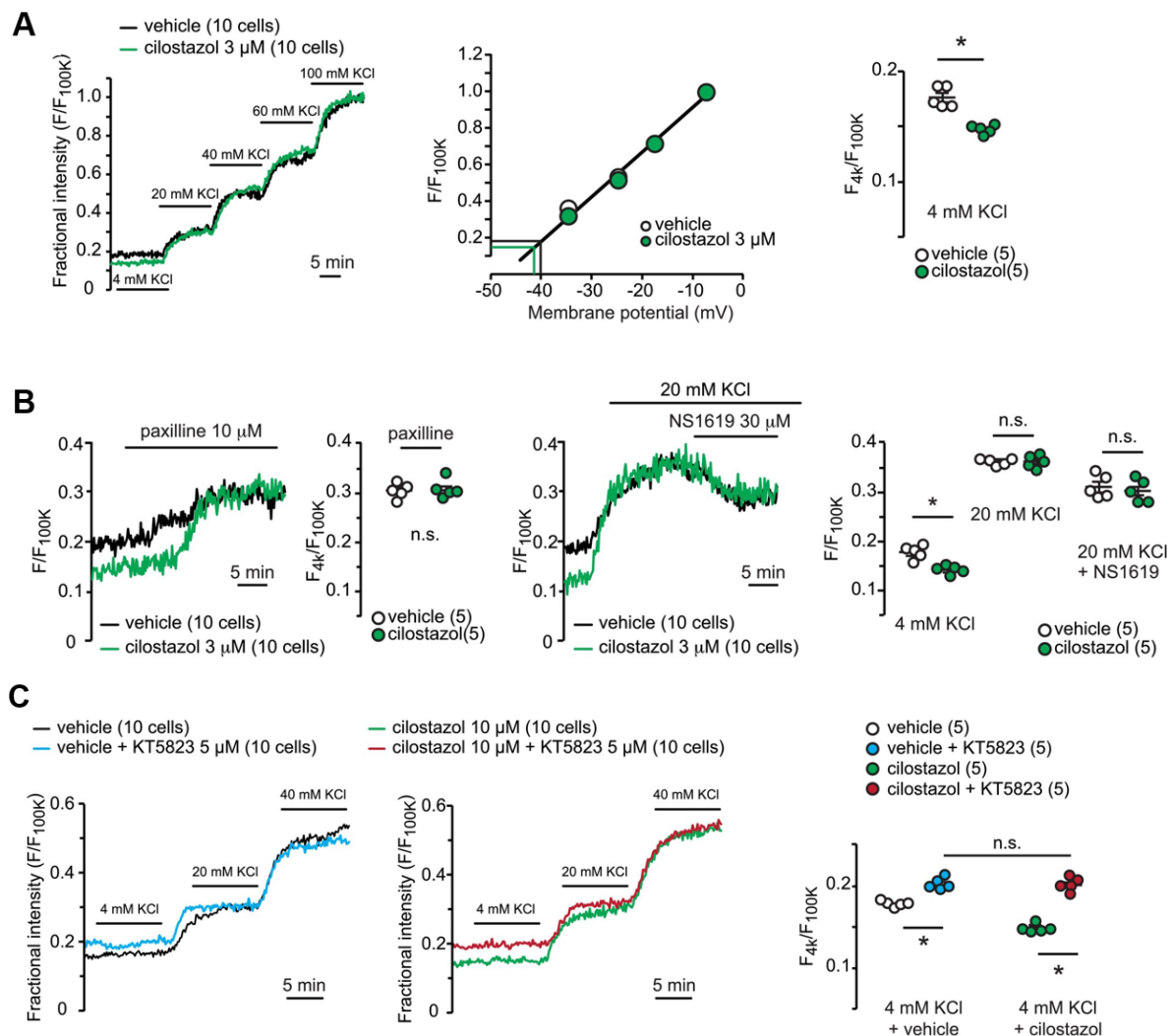


Figure 12. Cilostazol decreases membrane potential in growth plate chondrocytes.

(A) Confocal oxonol VI imaging of round chondrocytes. Femoral bone slices were pretreated with or without cilostazol for 30 min and then subjected to membrane potential imaging. During contiguous perfusion of high- K^+ solutions, cellular fluorescence intensities were monitored and normalized to the maximum value in the 100-mM KCl-containing solution to yield the fractional intensity. Representative averaged traces from 10 cells are shown (left panel). For preparation of the calibration plot (middle graph), the data from 10 cells in bathing solutions containing 4- (normal solution), 20-, 40-, 60- and 100-mM KCl are summarized; green and black lines indicate the estimated resting membrane potentials of the

cilostazol- and vehicle-pretreated cells, respectively. The resting fractional intensities were quantified and statistically analysed in the cilostazol- and vehicle-pretreated cells (right graph). The data are presented as the mean $\pm$ SEM, and the numbers of mice examined are shown in parentheses in the keys. Significant differences between the cilostazol- and vehicle-pretreated cells are indicated by asterisks ($*P < 0.05$, *t* test). (B) Effects of BK channel modulators on the resting membrane potential in round chondrocytes. The BK channel inhibitor paxilline abolished the difference between the vehicle- and cilostazol-treated cells (left panel and graph). The recording data from 10 cells pretreated with or without cilostazol were averaged and the fractional intensities elevated by paxilline are summarized. The BK channel activator NS1619 cancelled the difference between vehicle- and cilostazol-treated cells (right panel and graph). Recording data from 10 cells pretreated with or without cilostazol were averaged, and the fractional intensities in the normal, 20-mM KCl and NS1619-containing 20-mM KCl solutions are summarized. The data are presented as the mean $\pm$ SEM, the numbers of mice examined are shown in parentheses in the keys and significant differences between the cilostazol- and vehicle-pretreated cells are indicated by asterisks ($*P < 0.05$, *t* test). (C) Effect of the PKG inhibitor KT5823 on the resting membrane potential in round chondrocytes. After pretreatment with or without KT5823 and cilostazol, round chondrocytes in femoral bone slices were examined. In each pretreated group, the recording data of 10 cells were averaged (left panels), and the resting fractional intensities are summarized (right graph). The data are presented as the mean $\pm$ SEM, and the numbers of mice examined are shown in parentheses. Significant differences between each group are indicated by asterisks ($*P < 0.05$, two-way ANOVA and Sidak's test).

Cilostazol activates CaMKII in growth plate chondrocytes

CNP activates TRPM7-mediated Ca^{2+} entry and thus stimulates CaMKII to facilitate ECM formation in growth plate chondrocytes [13, 15]. Immunohistochemical analysis of round chondrocytes using an antibody against autophosphorylated CaMKII (pThr 286) revealed that the cilostazol-treated cells (3 μM for 30 min) were decorated more frequently and densely than the control cells (Figure 13A). The CaMKII inhibitor KN93 totally abolished the immunodecoration enhanced by cilostazol, indicating that the antibody precisely recognized the phosphorylated antigen. Furthermore, Western blot analysis of cell lysates prepared from

femoral growth plates detected that the cilostazol treatment significantly increased the phospho-CaMKII content without affecting the total CaMKII density (Figure 13B). Therefore, cilostazol seemed to activate CaMKII downstream of facilitated TRPM7-mediated Ca^{2+} entry in growth plate chondrocytes. Taken together with all the pharmacological data above, PDE3 inhibitors seemed to stimulate the cGMP-driven PKG-BK channel-TRPM7 channel-CaMKII signalling axis in the same manner as CNP.

We also examined the effect of PDE3 inhibitor treatments on the expression of genes associated with CNP signalling in cultured metatarsal bones treated with cilostazol or milrinone for 4 days, RT-PCR analysis detected reduced expression in *Prkg1*, *Prkg2*, *Camk2a* and *Camk2b* genes (Figure 14); the reduction rates ranged from 10% to 60%. Although these genes may be down-regulated under chronic cGMP-elevated conditions, PDE3 inhibitor treatments were unlikely to disrupt the CNP signalling pathway in growth plate chondrocytes.

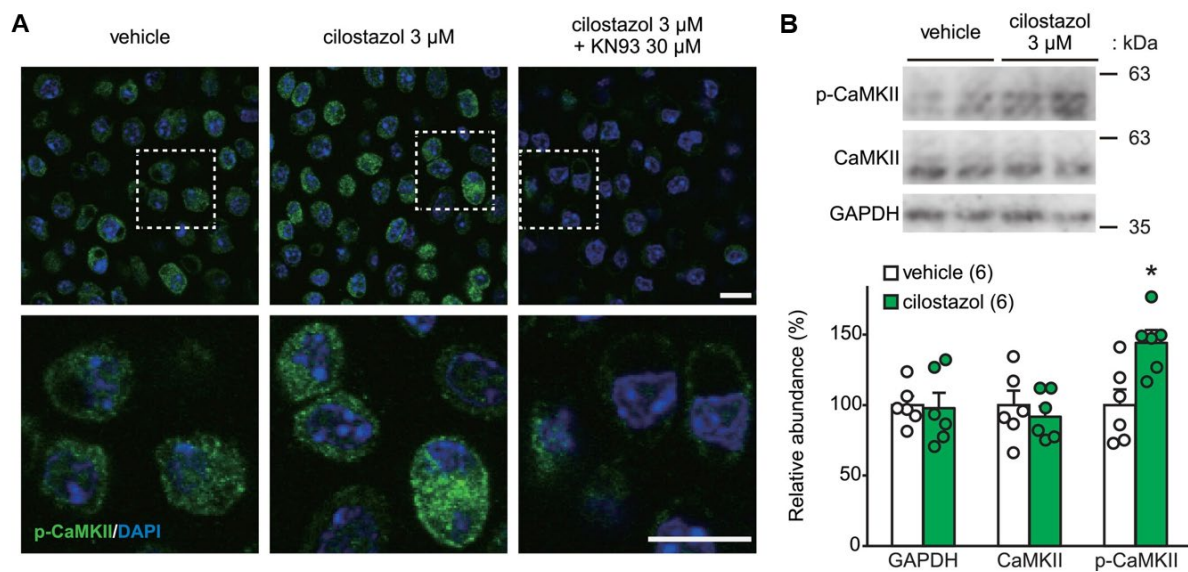


Figure 13. CaMKII activation in cilostazol-treated chondrocytes.

(A) Immunohistochemical staining for phospho-CaMKII (p-CaMKII) in round chondrocytes. Femoral bone slices were pretreated with or without cilostazol and the CaMKII inhibitor KN93 and then subjected to immunostaining with an antibody against p-CaMKII. 4',6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. The lower panels show high-magnification images of the white-dotted regions in the upper panels. Scale bars, 10 μ m. (B) Immunoblot analysis of CaMKII in growth plate cartilage. Growth plate lysates were prepared from femoral bone slices pretreated with or without cilostazol and subjected to immunoblot analysis with antibodies against total CaMKII and p-CaMKII (upper panel). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also analysed as a loading control. The immunoreactivities observed are summarized in the lower graph. The data are presented as the mean $\pm$ SEM, and the numbers of mice examined are shown in parentheses. A significant difference between the cilostazol- and vehicle-pretreated cells is indicated by an asterisk (* $P < 0.05$ according to t test).

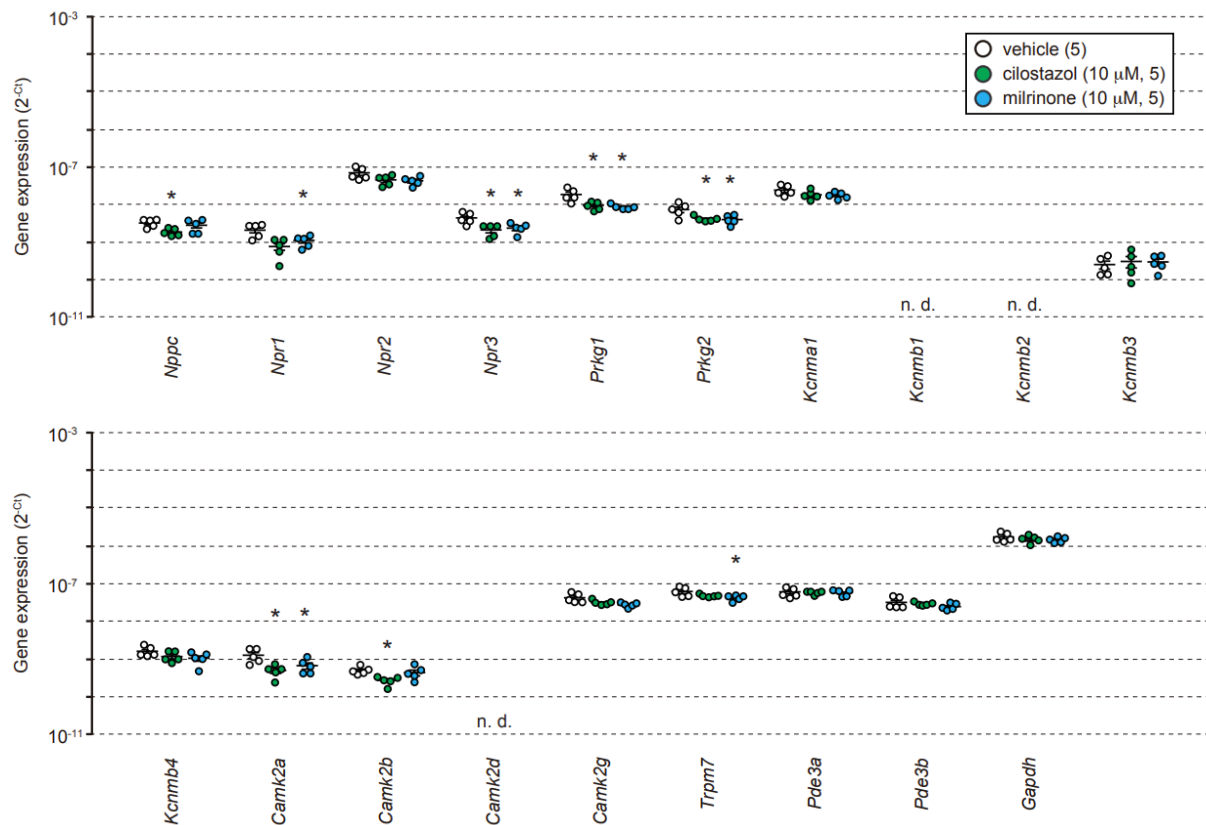


Figure 14. Gene expression analysis in PDE3 inhibitor-mediated bones.

RT-PCR analysis of genes related with CNP signalling in PDE3 inhibitor-treated metatarsal chondrocytes. The primer sets used are listed in Table 2 and the cycle threshold (Ct) was determined for each RT-PCR amplification. The data are presented as the mean $\pm$ SEM., and the numbers of mice examined are shown in parentheses.

Combined effects of CNP and cilostazol on cultured bone growth

Finally, we examined the combined effects of CNP and cilostazol on cultured bone elongation. Moderate (10 nM) and submaximal (30 nM) CNP concentrations resulted in increased of $127.6 \pm 0.6\%$ and $131.6 \pm 2.8\%$, respectively, in the longitudinal size of metatarsal bones during 4 days of culture. Cilostazol (10 μM) further stimulated bone outgrowth in 10-nM CNP-containing medium (Figure 15A) but had no effect on bone outgrowth in 30-nM CNP-containing medium (Figure 15B). Therefore, cilostazol seemed to

lose its pharmacological effect under high-CNP conditions. These observations were consistent with our conclusion that CNP and cilostazol stimulated the common signalling axis to promote bone growth.

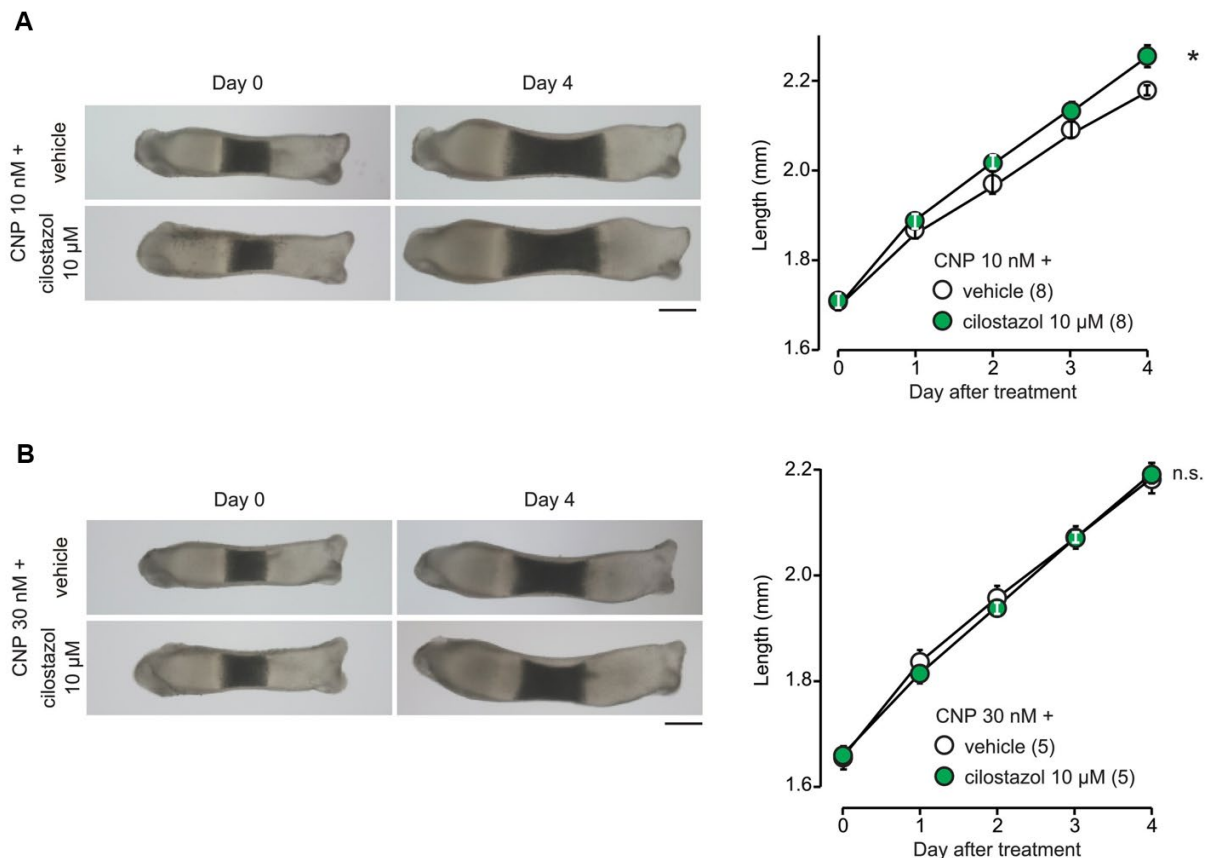


Figure 15. Combined effects of CNP and cilostazol on cultured bone outgrowth.

(A) Combined effects of 10-nM CNP and 10- μ M cilostazol. (B) Combined effects of 30-nM CNP and 10- μ M cilostazol. Representative images of the metatarsals before and after culture for 4 days under the indicated conditions are shown (left panels; scale bar, 0.3 mm). Longitudinal growth of the metatarsals was quantified over the culture period (right graphs). The numbers of mice examined are shown in parentheses. The bone sizes at the endpoint were analysed and a statistical difference from the vehicle-treated group is marked with an asterisk (* P < 0.05; n.s., not significant, two-way ANOVA and Sidak's test).

Discussion

In this study, we demonstrated that PDE3 inhibitors facilitate cultured bone outgrowth (Figure 3) and increase body size in juvenile mice (Figure 7). Our observations also suggest that cilostazol elevates cGMP levels, activates BK channels via PKG-catalysed phosphorylation and stimulates TRPM7-mediated Ca^{2+} entry by increasing the ion driving force (Figures 5, 6, 8), thus facilitating CaMKII (Figure 13) in growth plate chondrocytes. Therefore, PDE3 inhibitors probably enhance the CNP signalling cascade (Figure 16) and seem to stimulate ECM secretion downstream of CaMKII activation, leading to facilitation of bone elongation. This conclusion is further supported by the data that cilostazol did not stimulate the outgrowth of mutant bones from chondrocyte-specific Trpm7-knockout mice (Figure 6B).

In cilostazol-treated chondrocytes, we observed hyperpolarization of ~ 1.2 mV at a whole-cell level (Figure 12A) and 1.3-fold increase in the amplitude of spontaneous Ca^{2+} entry (Figure 9B). Such small potential shifts cannot definitively elevate Ca^{2+} amplitude by increasing the ionic driving force. However, it has been repeatedly reported in various cell types that slight hyperpolarizing events result in obvious functional changes in voltage-dependent channel gating, Ca^{2+} conduction and secretion. Local regions surrounding activated BK channels are more strongly hyperpolarized than observed in cell level analysis, because membrane voltage changes undergo exponential attenuation with distance and BK

channels are thought to often concentrate in specific microdomains [46]. Moreover, BK channels are reported to directly interact with several Ca^{2+} -permeable channels, such as Cav1.2, Cav2.1, Cav2.2, TRPC1 and NMDA receptor channels, to form functional nanodomains for channel crosstalk [47-49]. Based on the discrepancy between the weak hyperpolarization and facilitated Ca^{2+} entry, we could reasonably propose clusters of BK and TRPM7 channels in growth plate chondrocytes. Furthermore, it is a provocative hypothesis that both the channels, together with PLC, might concentrate to form Ca^{2+} -signalling platforms, because TRPM7 gating definitely depends on basal phosphoinositide turnover in growth plate chondrocytes [14].

In standard clinical treatments, cilostazol is used as an oral anti-platelet aggregation drug, while olprinone and milrinone are intravenously administered as inotropic and vasodilator drugs [50, 51]. Our data may predict that PDE3 inhibitors would be applicable for the treatment of various types of syndromes involving short stature via drug repositioning. The CNP analogue vosoritide has been clinically used via daily subcutaneous injection for achondroplasia treatment [11, 12]. Indication expansion trials using approved PDE3 inhibitors might provide patient-friendly oral drugs with economic benefits to combat various syndromes characterized by short stature. Moreover, based on the data from combined CNP and cilostazol treatments in bone culture (Figure 15), PDE3 inhibitors can be used in new combination therapies with vosoritide for achondroplasia patients.

The gene expression profile likely suggests that several PDE subtypes differentially contribute to the inactivation of cGMP and cAMP in growth plate chondrocytes (Figure 16). PDE3B seems to mainly maintain the intracellular cGMP concentration under resting conditions, because cilostazol and milrinone elevated the steady-state cGMP concentration in growth plate specimens (Figures 6 and 10). From a biochemical perspective, it is interesting to address the subtype-specific roles of the PDE species expressed in growth plate chondrocytes. Among the PDE species presumed to be expressed in the chondrocytes, PDE1B, PDE2A, PDE3B, PDE6G and PDE10A might differentially become active to hydrolyse cGMP and cAMP in response to various cellular conditions [33], and thus could modulate both the cGMP and cAMP signalling cascades. It has been reported that cAMP-dependent protein kinase (PKA), together with PKG, phosphorylates and activates BK channels in several cell types [52, 53]. Therefore, it is reasonably hypothesized that the CNP signalling cascade might be cooperatively controlled by PKG and PKA (Figure 16), both of which may be complexly regulated by the PDE subtypes in growth plate chondrocytes. To better understand the signal crosstalk proposed in CNP-induced long-bone outgrowth, investigation of the roles of the PKA and PDE subtypes would be further focused in developing growth plates.

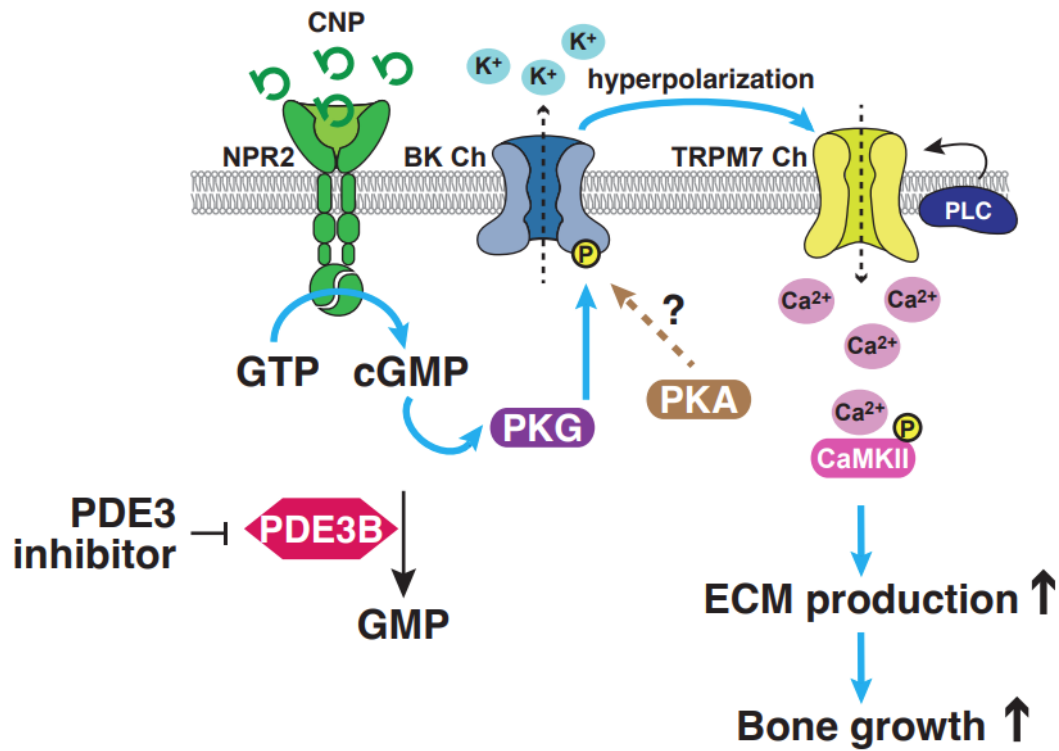


Figure 16. CNP signalling pathway and PDE3 inhibitors.

CNP stimulates the cGMP-driven PKG-BK channel-TRPM7 channel-CaMKII axis in growth plate chondrocytes and thus promotes bone growth by facilitating ECM synthesis [15]. Our present study suggested that PDE3 inhibitors facilitate the signalling axis by inhibiting cGMP hydrolysis.

Table 1. Reagents used in this study.

Reagents	supplier	identifier	dilution
Anti-phospho-CaMKII (Thr286) (Rabbit monoclonal)	Cell Signaling Technology	Cat#12716; RRID: AB_271389	WB (1/1000), IF (1/200)
Anti-CaMKII (Rabbit monoclonal)	Abcam	Cat#EP1829Y; RRID: AB_868841	WB (1/1000)
Anti-GAPDH (Rabbit polyclonal)	Sigma-Aldrich	Cat#G9545; RRID: AB_796208	WB (1/10000)
Anti-rabbit IgG-HRP (Mouse monoclonal)	Santa Cruz	Cat#sc-2357; RRID: AB_628497	WB (1/2000)
Anti-rabbit Alexa Fluor 488 (Goat polyclonal)	Invitrogen	Cat#A-11008; RRID: AB_143165	IF (1/500)
NaCl	Wako	Cat#191-01665	
KCl	Wako	Cat#163-03545	
CaCl ₂ ·2H ₂ O	Wako	Cat#033-25035	
MgCl ₂ ·6H ₂ O	Wako	Cat#135-00165	
Glucose	Wako	Cat#049-31165	
Glycine	Wako	Cat#077-00735	
2-Amino-2-hydroxymethyl-1,3-propanediol (Tris)	Nacalai tesque	Cat#35408-91	
2-Amino-2-Hydroxymethyl-1,3-propanediol hydrochloride (Tris-HCl)	Wako	Cat#012-17455	
Olive oil	Wako	Cat#150-00276	
HEPES	Dojindo	Cat#342-01375	
Methanol	Wako	Cat#139-01827	
Ethanol	Junsei	Cat#17065-0382	
Diethyl ether	Wako	Cat#052-01165	
Chloroform	Nacalai tesque	Cat#08402-55	
2-propanol	Nacalai tesque	Cat#29113-95	
Acetonitrile	Sigma-Aldrich	Cat # 34888	

Trifluoroacetic Acid	Wako	Cat#206-10731	
EDTA • 2Na	Dojindo	Cat#345-01865	
Trichloroacetic acid	Nacalai tesque	Cat#34603-02	
L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Nacalai tesque	Cat#13570-82	
β-glycerophosphate • 5H ₂ O pentahydrate	Wako	Cat#048-34332	
Penicillin-streptomycin solution	Wako	Cat#168-23191	
paraformaldehyde	Nacalai tesque	Cat#09154-85	
Mayer's hematoxylin solution	Wako	Cat#131-09665	
1 % eosin Y solution	Wako	Cat#051-06515	
Alcian-blue solution pH2.5	Merck Millipore	Cat#101647	
Super cryoembedding medium (SCEM)	Section-Lab		
MEM α	Thermo Fisher Scientific	Cat#12561056	
Albumin solution from bovine serum (fatty acid free)	Wako	Cat#017-22231	
Cilostazol	Tokyo Chemical Industry	Cat#C2587	
Milrinone	Tokyo Chemical Industry	Cat#M1663	
Anagrelide hydrochloride	Cayman	Cat#21411	
Olprinone hydrochloride	MedChemExpress	Cat#H-14254	
BAY-60-7550	Cayman	Cat#10011135	
MP10	Cayman	Cat#18266	
Fura-2 AM special packaging	Dojindo	Cat#F025	
Oxonol VI	AnaSpec	Cat#AS-84704	

FTY720	Sigma-Aldrich	Cat#SML0700	
Paxilline	Tocris Bioscience	Cat#2006/10	
KT5823	Cayman	Cat#10010965	
KN-93	Wako	Cat#115-00641	
Fetal bovine serum	Wako	Cat#533-69545	
Hyaluronidase from sheep testes	Sigma-Aldrich	Cat#H2126	
Sodium deoxycholate	Wako	Cat#192-08312	
NaF	Nacalai tesque	Cat#31420-82	
Na ₃ PO ₄ ·12H ₂ O	Nacalai tesque	Cat#31804-75	
Na ₃ VO ₄	Wako	Cat#198-09752	
Protease Inhibitor Cocktail for Use with Mammalian Cell and Tissue Extracts	Nacalai tesque	Cat#25955-24	
Sodium dodecyl sulfate	Wako	Cat#194-13985	
Acrylamide/Bis mixed solution	Nacalai tesque	Cat#06141-35	
WIDE RANGE Gel Preparation Buffer(4x) for PAGE	Nacalai tesque	Cat#07831-94	
TEMED	Nacalai tesque	Cat#33401-72	
Ammonium Peroxodisulfate	Wako	Cat#012-20503	
Blocking One	Nacalai tesque	Cat#03953-95	
ISOGEN	Nippon Gene	Cat#319-90211	
ReverTra Ace qPCR RT Master Mix with gDNA Remover	Toyobo	Cat#FSQ-301	
FastStart Essential DNA Green Master Mix	Roche	Cat#06924204001	
KOD FX	Toyobo	Cat#KFX-101X5	
Pierce BCA protein assay kit	Thermo Fisher Scientific	Cat#0023225	
cAMP ELISA kit	Enzo Life Sciences	Cat#ADI-900-066A	
cGMP ELISA kit	Enzo Life Sciences	Cat#ADI-900-014	

Table 2. Primers used in this study.

primer	forward	reverse
<i>Col2a1</i>	CACACTGGTAAGTGGGGCAAGACCG	GGATTGTGTTGTTTCAGGGTTCGGG
<i>Col10a1</i>	CAAGCCAGGCTATGGAAGTC	AGCTGGGCCAATATCTCCTT
<i>Pde1b</i>	GCAGCAGTTGGAAAGGATTG	TGTCACCCTGGCGGAAGAAC
<i>Pde2a</i>	ATCTTTGACCACTTCTCTCG	CATAACCCACTTCAGCCATC
<i>Pde3a</i>	AACTATACCTGCTCGGACTC	TTCGTGCGGCTTTATGCTGG
<i>Pde3b</i>	ATTCCAAAGCAGAGGTCATC	GTTAGAGAGCCAGCAGACAC
<i>Pde10a</i>	CATCCGCAAAGCCATCATCG	TCTCATCACCCCTCAGCCCAG
<i>Nppc</i>	CAGAAAAAGGGTGACAAGAC	CCTTCTTGTTGCCGCCTTTG
<i>Npr1</i>	AACAAGGAGAACAGCAGCAAC	TATCAAATGCCTCAGCCTGGA
<i>Npr2</i>	GGCCCCATCCCTGATGAAC	CCTGGTACCCCCTTCCTGTA
<i>Npr3</i>	GGTATGGGGACTTCTCTGTG	TCTGGTCTCATCTAGTCTCA
<i>Prkg1</i>	ATGGACTTTTTGTGGGACTC	GGTTTTCAATTGGATCTGGGC
<i>Prkg2</i>	TTGCGGAAGAAAATGATGTCG	GAATGGGGAGGTTGAGGAGAA
<i>Kcnma1</i>	AATGCACTTCGAGGAGGCTA	CTCAGCCGGTAAATTCCAAA
<i>Kcnmb1</i>	ACAACGTGTCTGCCCCCTCTA	CACTGTTGGTTTTGATCCCG
<i>Kcnmb2</i>	TCAGGAGACACCAACACTTC	AGTTAGTTTCACCATAGCAA
<i>Kcnmb3</i>	GTGGATGACGGGCTGGACTT	GCACTTGGGGTTGGTCCTGA
<i>Kcnmb4</i>	CTCCTGACCAACCCCAAGTG	TAAAATAGCAAGTGAATGGC
<i>Camk2a</i>	CACCACCATTGAGGACGAAG	GGTTCAAAGGCTGTCATTCC
<i>Camk2b</i>	AAGCAGATGGAGTCAAGCCC	TGCTGTCGGAAGATTCCAGG
<i>Camk2d</i>	GATAACAACAAAGCCAACG	GTAAGCCTCAAAGTCCCCAT
<i>Camk2g</i>	CAAGAACAGCAAGCCTATCC	CCTCTGACTGACTGGTGCGA
<i>Trpm7</i>	ATTGCTTAGTTTTGGTGTTT	GATTGTCGGGAGAGTGGAGT
<i>Gapdh</i>	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
<i>Trpm7 floxed</i>	TACATGTCTCTAGTCCAGCTTC	TACAAGGGCAAACCTGGAAGAC
<i>Col11Enh-cre</i>	CAATTTACTGACCGTACACCA	TCTTCAGGTTCTGCGGG
<i>Fgfr3-tg</i>	AGGTGGCCTTTGACACCTACCAGG	TCTGTTGTGTTTCCTCCCTGTTGG

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