Figure 6

Fig. 6

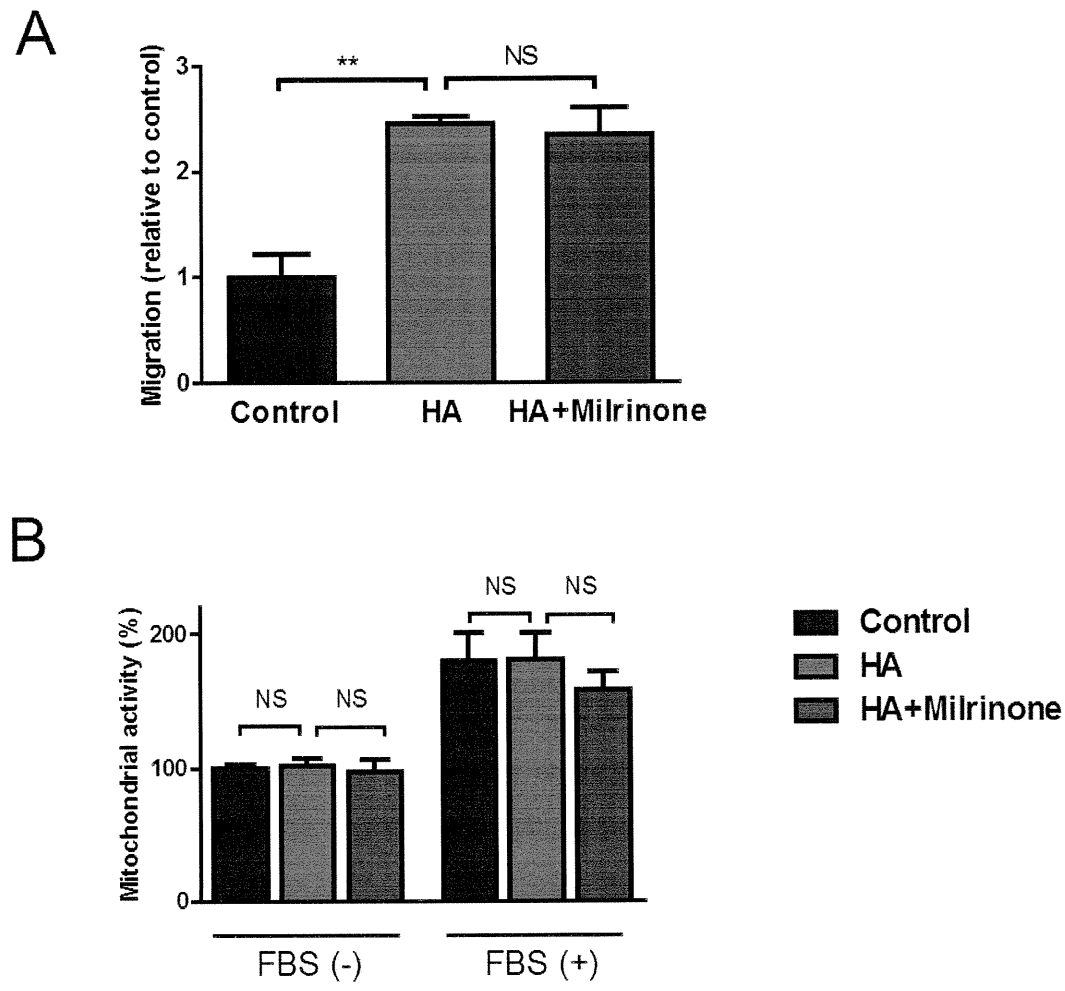


Figure 6　動脈管平滑筋細胞においてミルリノンとヒアルロン酸を両方投与したときの細胞遊走能および細胞増殖能。(A) ボイデンチャンバー法を用いてミルリノン (10 μM)＋ヒアルロン酸 (200ng/ml) で刺激したときの血管平滑筋の細胞遊走能 (n = 4)。(B) MTT アッセイでミルリノン (10 μM) ＋ヒアルロン酸 (200ng/ml) で刺激したときの細胞増殖能 (n = 8)。**$p < 0.01$. NS indicates not significant.

Figure 7

Fig. 7

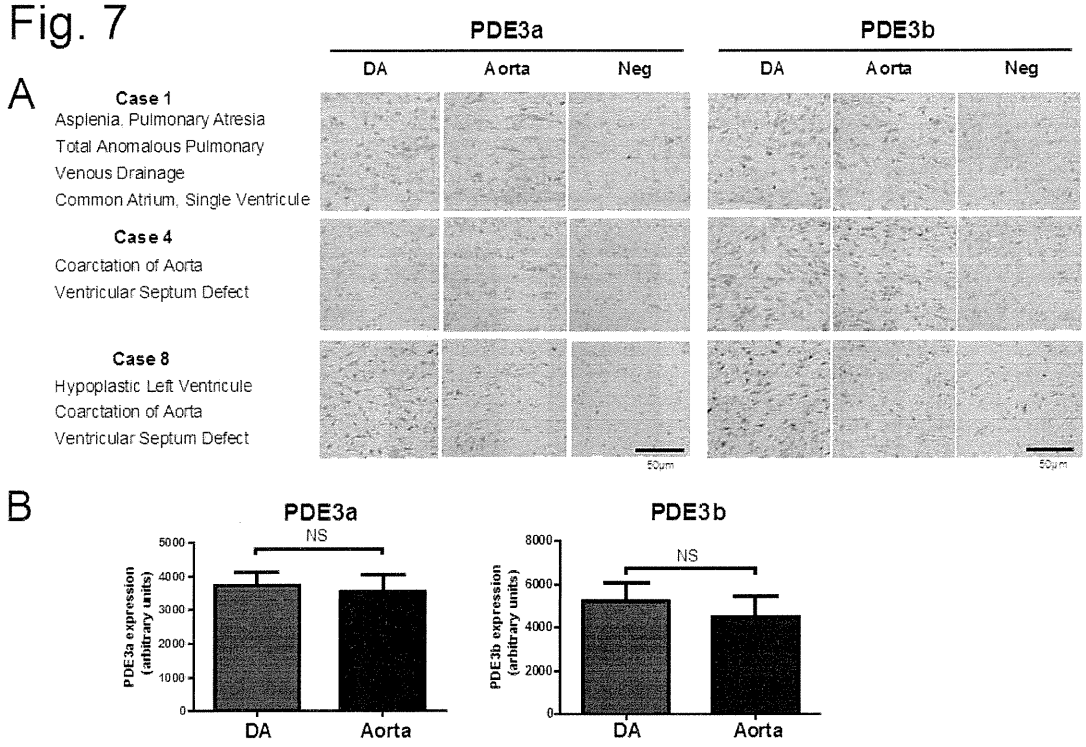


Figure 7　(A) さまざまな先天性心疾患における PDE3a と PDE3b の免疫染色。写真は動脈管、大動脈の平滑筋層である。PDE3a、PDE3b ともに一次抗体を使用しないと免疫反応は観察されない (Neg)。(B) 色抽出法を使用して、動脈管と大動脈の PDE3a と PDE3b の発現量を定量化した (n = 4)。NS indicates not significant.

Table 1　ヒト動脈管検体のプロフィール

Table 1.

Summary of patient characteristics

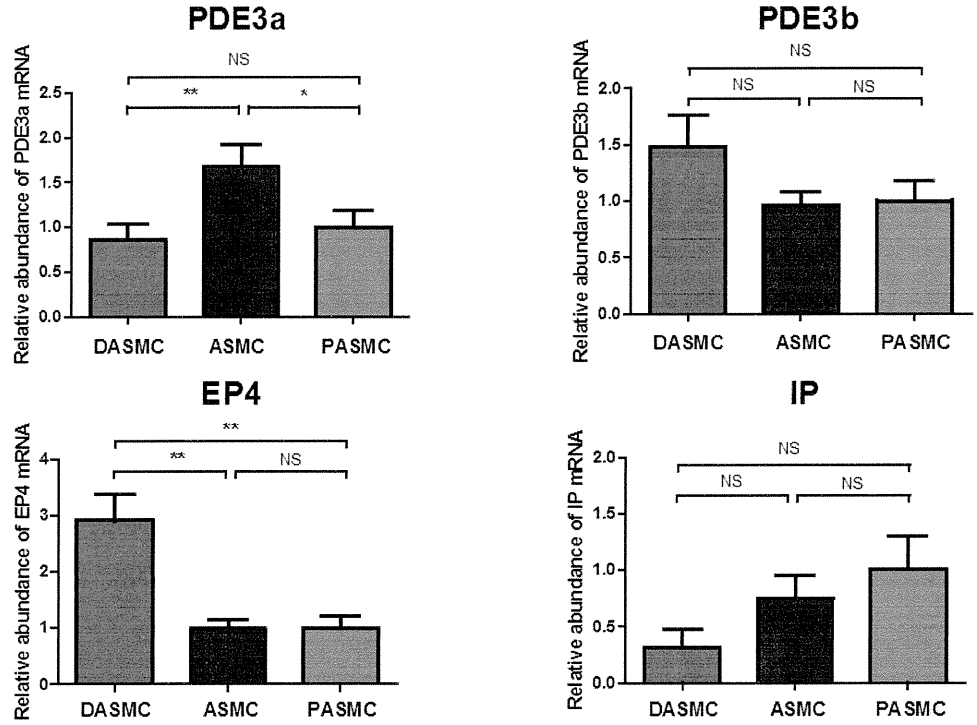
Case No.	Age at Operation	Diagnosis
1	0 days	Asplenia, PA, TAPVD, CA, SV
2	1 day	Asplenia, CoA, CA, SV
3	2 days	IAA, Aorticopulmonary window
4	2 days	CoA, VSD
5	3 days	TGA, CoA
6	4 days	CoA, VSD
7	13 days	CoA, VSD
8	1 month	hypoLV, CoA, VSD

PA: Pulmonary Atresia, TAPVD: Total Anomalous Pulmonary Venous Drainage,
CA: Common Atrium, SV: Single Ventricule,
CoA: Coarctation of Aorta, IAA: Interruption of Aortic Arch,
VSD: Ventricular Septum Defect, TGA: Transposition of the Great Arteries,
hypoLV: Hypoplastic Left Ventricule

PA:肺動脈閉鎖、TAPVD:総肺静脈還流異常症、CA:単心房、SV:単心室、CoA:大動脈縮窄、IAA:大動脈離断、VSD:心室中隔欠損症、TGA:完全大血管転移、hypoLV:左室低形成

補足 Figure 1

Supplemental Fig. S1



Supplemental Fig. S1

Quantitative RT-PCR of PDE3a, PDE3b, EP4 and IP in the DASMCs, ASMCs, PASMCs. All SMCs are used at passage 5 to 8. **P*<0.05, ***P*<0.01. NS: not significant. n=8.

ラット動脈管平滑筋細胞（DASMC）、大動脈平滑筋細胞（ASMC）、肺動脈平滑筋細胞(PASMC)における PDE3a、PDE3b、EP4 の定量 RT-PCR 解析。

[III]

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[IV]

研究成果の刊行物・別刷

Circulation Journal

Inhibition of Phosphodiesterase Type 3 Dilates the Rat Ductus Arteriosus Without Inducing Intimal Thickening

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Keywords:	Ductus arteriosus, Milrinone, Phosphodiesterase, congenital heart disease
Category:	Congenital heart desease/Child cardiovascular disease

Inhibition of Phosphodiesterase Type 3 Dilates the Rat Ductus Arteriosus Without Inducing Intimal Thickening

Yasuhiro Ichikawa MD [1,2], Utako Yokoyama MD, PhD [1], Mari Iwamoto MD, PhD [2], Jin Oshikawa MD, PhD [3], Satoshi Okumura MD, PhD [1], Motohiko Sato MD, PhD [1], Shumpei Yokota MD, PhD [2], Munetaka Masuda MD, PhD [4], Toshihide Asou MD, PhD [5], Yoshihiro Ishikawa MD, PhD [1]

1) Cardiovascular Research Institute, Yokohama City University
2) Department of Pediatrics, Yokohama City University
3) Department of Medical Science and Cardiorenal Medicine, Yokohama City University
4) Department of Surgery, Yokohama City University
5) Department of Cardiovascular Surgery, Kanagawa Children's Medical Center

Short title: PDE3 Inhibitors Dilate the DA Without Remodeling.

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Correspondence to: Utako Yokoyama or Yoshihiro Ishikawa, Cardiovascular Research Institute, Yokohama City University, 3-9 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan
Fax:+81-45-787-1470
Telephone:+81-45-787-2575
Email: utako@yokohama-cu.ac.jp or yishikaw@med.yokohama-cu.ac.jp
Total word count: 5812 words (7 figures and 1 table)

Abstract

Background: Prostaglandin E_1 (PGE_1), via cAMP, dilates the ductus arteriosus (DA). For patients with DA-dependent congenital heart diseases (CHDs), PGE_1 is the sole DA dilator that is used until surgery. However, PGE_1 has a short duration of action and frequently induces apnea. Most importantly, PGE_1 increases hyaluronan (HA) production, leading to intimal thickening (IT) and eventually DA stenosis after long-term use. In this study, therefore, we explored potential DA dilators, such as phophodiesterase 3 (PDE3) inhibitors, as alternatives to PGE_1.

Methods and Results: Expression levels of PDE3a and PDE3b mRNAs in rat DA tissues were higher than those in the PA. Intraperitoneal injection of milrinone (10 or 1 mg/kg) or olprinone (5 or 0.5 mg/kg) induced maximal dilatation of the DA lasting for up to 2 hours in rat neonates. In contrast, vasodilation induced by PGE_1 (10 μg/kg) was diminished within 2 hours. No respiratory distress was observed with milrinone or olprinone. Most important, milrinone did not induce HA production, cell migration, or proliferation when applied to cultured rat DA smooth muscle cells. Further, high expression levels of both PDE3a and PDE3b were demonstrated in the human DA tissues of CHD patients.

Conclusions: Because PDE3 inhibitors induced longer-lasting vasodilation without causing apnea or HA-mediated IT, they may be good alternatives to PGE_1 for patients with DA-dependent CHDs.

Word count: 217 words (abstract)

Key words: Ductus arteriosus, Milrinone, Phosphodiesterase, Congenital heart disease

Introduction

The ductus arteriosus (DA), the fetal arterial connection between the pulmonary artery and the descending aorta, is essential to maintain fetal life in utero. The DA closes after birth by two different mechanisms, namely, vasoconstriction and intimal thickening (IT) [1-3]. During the first few hours after birth, acute vasoconstriction occurs as a result of smooth muscle contraction in the DA. This is triggered by increased oxygen tension, due to the initiation of spontaneous breathing, and decreased circulating prostaglandin E_2 (PGE_2), due to disconnection from the placenta [3]. This functional vasoconstriction, however, must be preceded by intimal thickening of the DA, because vascular remodeling, including intimal thickening, is critical for anatomical closure of the DA.

The intimal thickening of DA is a result of many cellular processes, such as an increase in smooth muscle cell (SMC) migration and proliferation, the production of hyaluronan (HA) under the endothelial layer, and decreased elastin fiber assembly [1, 3, 4]. We have previously demonstrated that PGEs promoted HA production via cAMP/protein kinase A and subsequent SMC migration, resulting in intimal thickening of the DA during the late gestational period [1, 4, 5].

In patients with DA-dependent congenital heart diseases (CHDs), such as pulmonary atresia with intact ventricular septum or arch anomalies (coarctation of aorta or interruption of aortic arch), however, patent DA after birth is essential for survival. PGE_1 is widely used to keep the DA open as it increases intracellular cAMP and thus dilates the DA. However, PGE_1 induces hyaluronan (HA)-mediated intimal thickening and thus DA stenosis after prolonged use [6]. The fact that it induces only a very short duration of vasodilation, together with its severe adverse effects, such as apnea, respiratory distress, and hypotension, present additional problems, making it difficult for some patients with CHD to continue the use of PGE_1 until surgery. As such, possible alternatives to PGE_1 need to be explored.

Phosphodiesterases (PDEs), which catalyze the hydrolysis of cAMP/cGMP, constitute a superfamily of at least 11 gene families (PDE1–PDE11) [7]. The two PDE3 subfamilies, PDE3A and PDE3B, are encoded by closely related genes [8], and both hydrolyze cAMP. PDE3 inhibitors have been approved by the U.S. Food and Drug Administration (FDA) for use as vasodilators as well as in heart failure. Two of these are milrinone and olprinone, which are widely used to treat heart failure [9-12] and persistent pulmonary hypertension in neonates [13, 14]. Previous studies have shown that the PDE3 inhibitors milrinone, amrinone, and cilostazol counteract indomethacin-induced DA contraction [15, 16]. Thus, PDE3 inhibitors alone may be sufficient to dilate the DA. Nevertheless, it remains undetermined whether they induce intimal thickening, which is a major problem with PGE_1, via HA production, cell migration, or cell proliferation. In the current study, we investigated the role of PDE3 inhibitors in DA vascular remodeling and vasodilation with a view to their potential use as alternatives to the current PGE therapy.

Materials and Methods

Animals and materials

Timed pregnant Wistar rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All animal studies were approved by the institutional animal care and use committees of Yokohama City University. Milrinone, PDGF-BB, MTT, trichloroacetic acid, and 10% buffered formalin were obtained from Wako (Osaka, Japan). Olprinone, cilostazol, rolipram, PGE_1, PGE_2, elastase type II, trypsin inhibitor, bovine serum albumin V, poly-L-lysine, penicillin-streptomycin solution, acetic anhydride, triethylamine, Dulbecco's modified Eagle's medium (DMEM), and Hank's balanced salt solution (HBSS) were purchased from Sigma-Aldrich (St Louis, MO). Collagenase II was purchased from Worthington Biochemical Corp. (Lakewood, NJ). Collagenase/dispase

was purchased from Roche Diagnostics (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX).

Primary culture of rat smooth muscle cells (SMCs)

Vascular SMCs in primary culture were obtained from the DA (DASMCs), the aorta (ASMCs), and the pulmonary arteries (PASMCs) of Wistar rats on the 21st day of gestation. Isolation of DASMCs and ASMCs has been described previously [17]. To obtain PASMCs, the branch extralobular pulmonary arteries were dissected, cleaned from adherent tissue, and cut into small pieces. The tissues were transferred to a 1.5-ml centrifuge tube that contained 800 μl of collagenase-dispase enzyme mixture (1.5 mg/ml collagenase-dispase, 0.5 mg/ml of elastase type II-A, 1 mg/ml of trypsin inhibitor type I-S, and 2 mg/ml of bovine serum albumin fraction V in HBSS). Digestion was carried out at 37°C for 15 min. Cell suspensions were then centrifuged, and the medium was changed to a collagenase II enzyme mixture (1 mg/ml collagenase II, 0.3 mg/ml trypsin inhibitor type I-S, and 2 mg/ml bovine serum albumin fraction V in HBSS). After 12 min of incubation at 37°C, cell suspensions were transferred to growth medium in 35-mm poly-L-lysine-coated dishes in a moist tissue culture incubator at 37°C in 5% CO_2-95% ambient mixed air. The growth medium contained DMEM with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. We confirmed that >99% of cells were positive for α-smooth muscle actin and exhibited typical "hill-and-valley" morphology. Expression levels of PDE3, EP4, and prostacyclin (IP) receptor mRNAs in DASMCs, ASMCs, and PASMCs are shown in the Supplemental Fig. S1.

Human tissues from patients with CHDs

We obtained eight neonatal DAs and adjacent aortas during cardiac surgery in children between 0 days and 1 month of age. All excised tissue was fixed in 4% paraformaldehyde within 3 hours. The DA tissues were obtained from the Yokohama City University Hospital and Kanagawa Children's Medical Center. The study was

approved by the human subject committees at both Yokohama City University and Kanagawa Children's Medical Center. Detailed patient information is summarized in Table 1.

RNA isolation and quantitative RT-PCR

Pooled vascular tissues were obtained from Wistar rats on the 21st day of gestation. After excision, tissues were frozen in liquid nitrogen and stored at -80°C. The total RNA was isolated from the tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and from the cultures using Trizol reagent (Invitrogen, Carlsbad, CA). The primers were designed based on the rat nucleotide sequences of PDE3a (NM_017337) (5'- CGC CTG AGA AGA AGT TTG C -3' and 5'- AGA CAG CAT AGG ACG AAG TGA AG -3'), PDE3b (NM_017229.1) (5'- TCC AAA GCA GAG GTC ATC ATC -3' and 5'- GTA TCA AGA AAT CCT ACG GGT GA -3'), EP4 (NR_032076.3) (5'- CTC GTG GTG CGA GTG TTC AT -3' and 5'- AAG CAA TTC TGA TGG CCT GC -3'), and IP (NM_00177644.1) (5'- GGG CAC GAG AGG ATG AAG -3' and 5'- GGG CAC ACA GAC AAC ACA AC -3'). Reverse transcription polymerase chain reaction (PCR) was performed using a PrimeScript RT reagent Kit (TaKaRa Bio, Tokyo, Japan) and real-time PCR was performed using SYBR Green (Applied Biosystems, Foster City, CA). The abundance of each gene was determined relative to that in 18S ribosomal RNA.

Rapid whole-body freezing method

To study the in situ morphology and inner diameter of the neonatal DA, a rapid whole-body freezing method was used as previously described [2]. Fetuses on the 21st day of gestation were delivered by cesarean section and intraperitoneally injected immediately after birth with milrinone (10 mg/kg, 1 mg/kg, 0.1 mg/kg), olprinone (5 mg/kg, 0.5 mg/kg, 0.05 mg/kg), or PGE_1 (10 μg/kg). The rat pups were frozen in liquid nitrogen at 0, 0.5, 1, 2, 4, 6, 8, and 12 hours after injection. The frozen thoraxes were

then cut on a microtome, and the inner diameter of each DA was measured.

Determination of respiratory rate

Fetuses on the 21st day of gestation were delivered by cesarean section and intraperitoneally injected 0 or 2 hours after birth with milrinone (10 mg/kg, 1 mg/kg), olprinone (5 mg/kg, 0.5 mg/kg), or PGE_1 (10 μg/kg). We measured the respiratory rate by counting the movements of the rat thorax.

Quantitation of hyaluronan (HA)

The amount of HA in the cell culture supernatant was measured according to the latex agglutination method as previously described [1].

SMC migration assay

The migration assay was performed using 24-well transwell culture inserts with polycarbonate membranes (8-μm pores) (Corning Inc., Corning, NY) as previously described [1]. Cells were stimulated with milrinone (10 μM), PGE1 (1 μM), PDEF-BB (10 ng/ml), HA (200 ng/ml), or milrinone+HA for 3 days.

Cell proliferation assay

SMCs were cultured on 24-well plates at 1×10^5 cells per well in DMEM supplemented with 10% FBS. After various treatments over 3 days, 500 μl of 1 mg/ml MTT solution was added to each well and incubated for 2 hours. The supernatants were aspirated, and the formazan crystals in each well were solubilized with 0.05 M HCl (500 μl). Each solution (100 μl) was placed in a 96-well plate. SMC proliferation was measured based on absorbance at 570 nm using a microplate reader.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described [1, 18]. Rabbit polyclonal anti-PDE3A antibody (sc-20792) and goat polyclonal anti-PDE3b antibody (sc-11835) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A color extraction method using BIOREVO bz-9000 (KEYENCE, Osaka, Japan) was performed

to quantify the expression of PDE3s in the DAs and the aortas of case 1, 4, 5, and 8 (Table 1). Eighteen fields in the smooth muscle layer of the DA and the aorta respectively were examined in four cases. Diaminobenzidine (DAB)-stained colors, PDE3a-positive or PDE3b-positive areas, were extracted and counted on the screen.

Cyclic AMP Production Measured by Radioimmunoassay

Measurement of cAMP accumulation in DASMCs was performed as previously described [2, 19]. Briefly, DASMCs grown on 24-well plates were serum-starved for 24 h and assayed for cAMP production after a 10- or 20-min period of incubation with 10 μM of milrinone. Reactions were terminated by aspiration of the media and the addition of 300 μl of ice-cold trichloroacetic acid (7.5%) to each well. Forty microliters of each sample were acetylated and incubated with ^{125}I-cAMP (Perkin Elmer, Waltham, MA) and 50 μl of rabbit anti-cAMP antibody (diluted 1:3000, Millipore, Billerica, MA) overnight at 4°C. Each mixture was then incubated with 50 μl of goat anti-rabbit antibody with magnetic beads (Qiagen, Valencia, CA) for 1 h. Separation of bound antibodies from free antibodies was achieved by filtration, and bound radioactivity was counted. Production of cAMP was normalized to the amount of protein per sample.

Statistics

Data are presented as means ± standard error of the mean (SEM) of independent experiments. Statistical analysis was performed between two groups by unpaired two-tailed Student's *t* test or unpaired *t* test with Welch's correction, and among multiple groups by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A p value of <0.05 was considered significant.

Results

Messenger RNA of PDE3 isoforms was highly expressed in rat DA

We first examined whether the target molecule of PDE3 inhibitors is highly expressed in the DA. We measured the mRNA expression levels of PDE3s using quantitative RT-PCR in the rat DA, aorta, and pulmonary arteries (PA) on the 21st day of gestation (Figure 1). Expression of PDE3a mRNA was higher in the DA than in the PA. Expression of PDE3b mRNA was higher in the DA than in the aorta or the PA. We also confirmed that EP4 mRNA was more highly expressed in the DA than in the aorta or the PA. Thus, PDE3 isoforms were abundantly expressed in the DA relative to the PA.

Vasodilatory effects of PDE3 inhibitors on rat DA in vivo

PDE3 inhibitors are widely used in neonates and children with low cardiac output following myocarditis and cardiovascular surgery for congenital heart disease [20, 21]. We examined whether milrinone or olprinone dilated the DA using the rapid whole-body freezing method in rat neonates. Neonates were injected with one of these drugs immediately after birth to mimic the vasodilatory treatment currently used in DA-dependent congenital heart diseases.

Intraperitoneal injection of PGE_1 (10 μg/kg, the amount that is intravenously administered daily as a clinical maintenance dose) induced maximal dilatation of the DA for 30 min, but this effect was completely lost within 2 h after injection (Figure 2A). A single intraperitoneal administration of 10 mg/kg of milrinone maintained maximal dilation of the DA for up to 12 h (Figures 2B, 2C). 1 mg/kg of milrinone, the amount that is intravenously administered daily as a clinical maintenance dose, maintained maximal dilatation for 2 h, after which DA closure occurred at 4 h after injection. 0.1 mg/kg of milrinone did not affect DA tone. Both 5 mg/kg and 0.5 mg/kg of olprinone, the latter of which is suitable for daily intravenous administration as a clinical maintenance dose, induced maximal dilatation for 1 h after injection (Figures 2D, 2E). 0.05 mg/kg of olprinone did not dilate the DA. Thus, both milrinone and olprinone produced dose-dependent vasodilatory effects (Figure 2F), but those of milrinone lasted