Prostaglandin E\textsubscript{2}-activated Epac Promotes Neointimal Formation of the Rat Ductus Arteriosus by a Process Distinct from That of cAMP-dependent Protein Kinase A\textsuperscript{*}

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Utako Yokoyama\textsuperscript{†1}, Susumu Minamisawa\textsuperscript{†1+2}, Hong Quan\textsuperscript{†1}, Toru Akaike\textsuperscript{†1}, Sayaka Suzuki\textsuperscript{†1}, Meihua Jin\textsuperscript{†1}, Qibin Jiao\textsuperscript{†1}, Mayumi Watanabe\textsuperscript{†1}, Koji Otsu\textsuperscript{†1}, Shiho Iwasaki\textsuperscript{†*1}, Shigeru Nishimaki\textsuperscript{†*1}, Motohiko Sato\textsuperscript{†1}, and Yoshihiro Ishikawa\textsuperscript{†1+1}

From the \textsuperscript{†}Cardiovascular Research Institute and the \textsuperscript{**}Department of Pediatrics, Yokohama City University Graduate School of Medicine, Kanagawa 236-0027, Japan, the \textsuperscript{‡}Institute for Biomedical Engineering, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo 162-8480, Japan, the \textsuperscript{¶}Department of Life Science and Medical Bio-science, Waseda University Graduate School of Advanced Science and Engineering, Tokyo 162-8480, Japan, and the \textsuperscript{‡}Cardiovascular Research Institute, Departments of Cell Biology and Molecular Medicine and Medicine (Cardiology), New Jersey Medical School, Newark, New Jersey 07101-1709

We have demonstrated that chronic stimulation of the prostaglandin E\textsubscript{2}-cAMP-dependent protein kinase A (PKA) signal pathway plays a critical role in intimal cushion formation in perinatal ductus arteriosus (DA) through promoting synthesis of hyaluronan. We hypothesized that Epac, a newly identified effector of cAMP, may play a role in intimal cushion formation (ICF) in the DA distinct from that of PKA. In the present study, we found that the levels of Epac1 and Epac2 mRNAs were significantly up-regulated in the rat DA during the perinatal period. A specific EP4 agonist, ONO-AE1–329, increased Rap1 activity in the presence of a PKA inhibitor, PKI-(14–22)-amide, in DA smooth muscle cells. 8-p-cPTP-2’-O-Me-cAMP (O-Me-cAMP), a cAMP analog selective to Epac activator, promoted migration of DA smooth muscle cells (SMC) in a dose-dependent manner. Adenovirus-mediated Epac1 or Epac2 gene transfer further enhanced O-Me-cAMP-induced cell migration, although the effect of Epac1 overexpression on cell migration was stronger than that of Epac2. In addition, transfection of small interfering RNAs for Epac1, but not Epac2, significantly inhibited serum-mediated migration of DA SMCs. In the presence of O-Me-cAMP, actin stress fibers were well organized with enhanced focal adhesion, and cell shape was widely expanded. Adenovirus-mediated Epac1, but not Epac2 gene transfer, induced prominent ICF in the rat DA explants when compared with those with green fluorescent protein gene transfer. The thickness of intimal cushion became significantly greater (1.98-fold) in Epac1-overexpressed DA. O-Me-cAMP did not change hyaluronan production, although it decreased proliferation of DA SMCs. The present study demonstrated that Epac, especially Epac1, plays an important role in promoting SMC migration and thereby ICF in the rat DA.

Prostaglandin E\textsubscript{2} (PGE\textsubscript{2})\textsuperscript{3} is the most potent vasodilatory lipid mediator in the ductus arteriosus (DA), a fetal arterial connection between the pulmonary artery and the descending aorta (1). PGE\textsubscript{2} increases the intracellular concentration of cAMP, which activates cAMP-dependent protein kinase A (PKA), resulting in vasodilation in the DA (1, 2). In addition to its vasodilatory effect, our recent study has identified that chronic PGE\textsubscript{2} stimulation has another essential effect on DA development, namely intimal cushion formation (ICF) (3). Briefly, via EP4, a predominant PGE\textsubscript{2} receptor in the DA, the PGE\textsubscript{2}-cAMP-PKA stimulation up-regulates hyaluronic acid (HA) synthases, which increases HA production. Accumulation of HA then promotes smooth muscle cell (SMC) migration into the subendothelial layer to form intimal thickening. ICF then leads to luminal narrowing, helping adhesive occlusion of the vascular lumen and thus complete anatomical closure of the DA.

A new target of cAMP, i.e. an exchange protein activated by cAMP, has recently been discovered; it is called Epac (4).

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\textsuperscript{1} These authors contributed equally to this work.

\textsuperscript{2} To whom correspondence should be addressed: Dept. of Life Science and Medical Bio-science, Waseda University Graduate School of Advanced Science and Engineering 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan. Tel: 81-3-5369-7322; Fax: 81-3-5369-7022; E-mail: saminamis@waseda.jp.

\textsuperscript{3} The abbreviations used are: PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; PKA, cAMP-dependent protein kinase A; DA, ductus arteriosus; PKI-(14–22)-amide, PKA inhibitor-(14–22)-amide; O-Me-cAMP, 8-p-cPTP-2’-O-Me-cAMP; pCTP-cAMP, 4-chlorophenylthio)-adenosine 3’5’-cyclic monophosphate cAMP; ICF, intimal cushion formation; HA, hyaluronic acid; SMC, smooth muscle cell; RT-PCR, reverse transcription-PCR; PBS, phosphate-buffered saline; GFP, green fluorescent protein; siRNA, small interfering RNA.
Epac has been known to exhibit a distinct cAMP signaling pathway that is independent of PKA activation (4). Epac is a guanine nucleotide exchange protein that regulates the activity of small G proteins. There are two variants; Epac1 was found to be expressed in most tissues including the heart and blood vessels, whereas Epac2 is expressed in the adrenal gland and the brain (4). Various roles have been proposed for Epac, such as cell proliferation or transformation, but the role of the Epac signal in cardiovascular pathophysiology remains poorly understood. Since PGE$_2$ accumulates intracellular cAMP in the DA during late gestation, it is reasonable to assume that PGE$_2$ activates not only PKA but also Epac pathways in the DA. Therefore, we hypothesized that Epac played a role distinct from that of PKA in vascular remodeling in the DA, especially in the ICF process.

EXPERIMENTAL PROCEDURES

Animals and Tissue—Timed-pregnant Wistar rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). The DA was obtained from rat fetuses on the 19th day of gestation (preterm) and on the 21st day of gestation (term), as well as from neonates within 6 h after delivery. On the 19th day of gestation, the DA was still immature; it became mature on the 21st day of gestation. All animals were cared for in compliance with the guiding principles of the American Physiological Society. The experiments were approved by the Ethical Committee of Animal Experiments at the Yokohama City University School of Medicine.

Quantitative Reverse Transcription (RT)-PCR—Isolation of total RNA, generation of cDNA, and quantitative RT-PCR analysis were performed as described previously (3, 5). Rat Epac1 primers (Rn00572463_m1) and TaqMan rodent GAPDH primers for PCR amplification were purchased from Applied Biosystems Inc. (Foster City, CA). For detection of human Epac1, the primers were used as described previously (6). The sequences of rat Epac2 primers were 5'-atc aga tta cgc gat gaa-3' (forward) and 5'-aag ctt gcg cca aag cac ctg cca aag cac ctg (reverse) (Invitrogen). We confirmed that the sequence of the PCR product amplified by the rat Epac2 primers was only from Epac2 cDNA. We confirmed that both Epac1 and Epac2 primers were similarly efficient to amplify each PCR product. The abundance of each gene was determined relative to GAPDH.

Primary Culture of Rat DA SMCs—Vascular SMCs in primary culture were obtained from the DA as described previously (3, 5). The confluent cells between passages 4 and 6 were used in the experiments.

Immunohistochemistry—Paraffin-embedded blocks containing DA tissues were prepared as described previously (5); tissue staining and immunohistochemistry were performed as described previously (3, 5). Antibodies of Epac1 and Epac2 were purchased from Upstate Biotechnology and Santa Cruz Biotechnology. There was little cross-reaction between Epac1 and Epac2 antibodies. Anti-αSMA antibody was obtained from Sigma-Aldrich. Anti-Ki67 and anti-von Willebrand factor antibodies were from Dako. DA SMCs cultured on 12-mm glass coverslips were serum-starved for 24 h and then stimulated for 1 h in medium alone (control) or with ONO-AE1–329 (an EP4-specific agonist, 10$^{-8}$–10$^{-6}$ M), O-Me-cAMP (an Epac-specific agonist, 30 μM), or pCPT-cAMP (a PKA-specific agonist, 500 μM). ONO-AE1–329 was kindly provided by Ono Pharmaceutical Inc. O-Me-cAMP and pCPT-cAMP were purchased from Sigma-Aldrich. Cells were then fixed in 10% buffered formalin for 10 min, washed twice with PBS, and permeabilized in 0.3% Triton X-100 in PBS for 10 min. DA SMCs were washed twice with (0.1% Tween) in PBS and incubated with 1% bovine serum albumin, 0.1% Tween 20 in PBS for 20 min and then with an rhodamine-conjugated phallolidin antibody (Invitrogen) and paxillin antibody (BD Biosciences) for 16 h at 4°C. Some specimens with a primary antibody were prepared without staining DA tissues were prepared as described previously (5); tissue staining and immunohistochemistry were performed as described previously (3, 5). Rat tissue specimens with a primary antibody were prepared without staining.
incubation as negative controls. An additional 60-min incubation was carried out with a secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). Nuclear staining was achieved with 4',6-diamidino-2-phenylindole (Invitrogen). After two washes with 0.1% Tween 20 in PBS, coverslips were mounted for microscopic imaging. All incubation and wash steps were conducted at room temperature. Morphometric analyses were performed using Nikon TE2000-E (Tokyo, Japan).

**Rap1 Activation Assay**—DA SMCs were grown to 70% confluence, serum-starved for 24 h, and then stimulated for 15 min in medium alone (control) or with ONO-AE1–329 (10^{-6} M), O-Me-cAMP (30 μM), pCPT-cAMP (500 μM), or a PKA inhibitor, PKI-(14–22)-amide (10^{-5} M). PKI-(14–22)-amide was purchased from Calbiochem. Rap1 activity was measured using EZ-Detect RAP1 activation kit (Pierce) according to the manufacturer’s protocol (6).

**SMC Migration Assay**—The migration assay was performed using 24-well Transwell culture inserts with polycarbonate membranes (8-μm pores; Corning) as described previously (3) with fibronectin coating.

**Adenovirus Construction**—For construction of adenoviral vectors, full-length cDNA-encoding human Epac1 or Epac2 was cloned into an adenoviral vector by using an AdenoX adenovirus construction kit (Clontech). Both of the cDNAs were kindly provided by Dr. J. L. Bos at University Medical Center, Utrecht, The Netherlands. Adenovirus-mediated transduction was performed as described elsewhere (7). As a control study, adenovirus vector harboring green fluorescent protein (GFP) was used at the same multiplicity of infection.

**RNA Interference (siRNA)**—Two double-stranded 21-bp small interfering RNAs (siRNAs) to the selected region of Epac1 cDNA or Epac2 cDNA were purchased from Ambion Inc. or Qiagen Inc., respectively. The antisense siRNA sequences targeting Epac1 were 5’-gucc tgt-3’ and 5’-gag aac gua cag gag c(t)-3’. The antisense siRNA sequences targeting Epac2 were 5’-uag aau guc guu gaa ugc c(tt)-3’ and 5’-uua auc acu aca ucc g(at)-3’. AllStars negative control siRNA (Qiagen) was used as a control nonsilencing siRNA. DA SMCs were serum-deprived and transfected with siRNA (100 pmol) using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the instructions of the manufacturer. Cells were kept serum-free for an additional 24 h before the experiments.

**Quantitation of HA**—The amount of HA in the cell culture supernatant was measured by a latex agglutination method, described previously (3).

**SMC Proliferation Assay**—The proliferation assay was performed by measurement of [3H]thymidine incorporation as described previously (3). Briefly, the DA SMCs were reseeded into a 24-well culture plate at an initial density of 1 × 10^5 cells/well for 24 h before serum starvation with Dulbecco’s modified Eagle’s medium containing 0.5% fetal calf serum. Cells were then incubated with or without O-Me-cAMP (50 μM) for 24 h in the starvation media before adding 1 μCi [3H]methyl thymidine (specific activity 5 Ci/mmol/liter; Amersham Biosciences International) for 4 h at 37 °C. Data obtained from triplicate wells were averaged.

![FIGURE 2. Rap1 was activated by Epac or EP4 stimulation with PKA inhibition. A, Rap1 activity assay revealed that O-Me-cAMP (30 μM), a cAMP analog selective to Epac (8), strongly increased Rap1 activity in DA SMCs, whereas pCPT-cAMP (500 μM), a CAMP analog selective to PKA or a specific EP4 agonist, ONO-AE1–329 (10^{-6} M), did not. O-Me-cAMP-mediated activation of Rap1 was antagonized by pCPT-cAMP, n = 4–8. B, in the presence of a PKA inhibitor, PKI-(14–22)-amide (10^{-5} M), ONO-AE1–329 (10^{-6} M) increased Rap1 activity, n = 3–4. The values are expressed as mean ± S.E. ** indicates p < 0.01 to control.](image-url)
analysis of variance followed by Newman-Keuls’ multiple comparison test. A value of \( p < 0.05 \) was considered significant.

**RESULTS**

The Expression of Epac1 and Epac2 in the Rat DA—Quantitative RT-PCR analysis revealed that the expression levels of Epac1 and Epac2 mRNAs increased significantly with development and reached their maximum on the day of birth (Fig. 1, A and B). The relative expression levels of Epac2 mRNA were approximately two times higher than those of Epac1 mRNA in a perinatal period. Immunohistological analysis showed that the expression patterns of Epac1 and Epac2 proteins in the rat DA were similar (Fig. 1C). They were faintly expressed in the smooth muscle layer of the immature DA on the 19th day of gestation. The stains of both Epac1 and Epac2 proteins became strong, especially in the endothelial and subendothelial layers, in the mature DA on the 21st day of gestation. After birth, the DA lumen was filled with endothelial cells and migrated SMCs (supplemental Fig. 1). Epac1 and Epac2 proteins were predominantly stained in these cells of the central core of the DA lumen.

**EP4 Stimulation with PKA Inhibition Increased Rap1 Activity**—We examined whether PGE\(_2\)-EP4 activates not only cAMP-PKA but also the cAMP-Epac pathway in DA SMCs by Rap1 activity assay. As expected, O-Me-cAMP, a cAMP analog selective to Epac (8), strongly increased Rap1 activity, whereas pCPT-cAMP, a cAMP analog selective to PKA, did not (Fig. 2A). We found that the Epac-stimulated Rap1 activity was almost abolished by adding pCPT-cAMP, suggesting that PKA antagonized the effect of Epac on Rap1 activity.

PKA but also the cAMP-Epac pathway in DA SMCs by Rap1 activity assay. As expected, O-Me-cAMP, a cAMP analog selective to Epac (8), strongly increased Rap1 activity, whereas pCPT-cAMP, a cAMP analog selective to PKA, did not (Fig. 2A). We found that the Epac-stimulated Rap1 activity was almost abolished by adding pCPT-cAMP, suggesting that PKA antagonized the effect of Epac on Rap1 activity. A selective EP4 agonist, ONO-AE1–329 (10^{-6} M), did not increase Rap1 activity (Fig. 2A). Then, we tested whether ONO-AE1–329 indeed did not activate the Epac-Rap1 pathway in DA SMCs or the effect was masked by simultaneous activation of PKA. In the presence of a PKA inhibitor, PKI-(14–22)-amide, ONO-AE1–329 increased Rap1 activity to a similar extent to O-Me-cAMP (Fig. 2B). These data indicated that EP4 activated not only cAMP-PKA but also cAMP-Epac pathway in DA SMCs.
**Epac Promoted DA SMC Migration**—When DA SMCs were exposed to O-Me-cAMP for 4 h, O-Me-cAMP promoted DA SMC migration in a dose-dependent manner (Fig. 3A). On the contrary, acute stimulation (4 h) of pCPT-cAMP significantly inhibited DA SMC migration (Fig. 3A), which is consistent with previous studies on other vascular SMCs (9, 10). When DA SMCs were exposed to both O-Me-cAMP (50 μM) and pCPT-cAMP (500 μM) for 4 h, DA SMC migration was significantly inhibited, indicating that Epac-stimulated cell migration was completely antagonized by PKA stimulation.

When Epac1 or Epac2 was overexpressed by adenovirus-mediated gene transfer using the same titer of adenoviral vectors, the effect of O-Me-cAMP on DA SMC migration was further enhanced when compared with control LacZ overexpression (Fig. 3B). However, the effect of Epac1 overexpression on cell migration was much stronger than that of Epac2 overexpression.

Furthermore, since no selective Epac inhibitor is available so far, we prepared two different siRNAs for Epac1 or Epac2 to examine whether a decrease in the expression of Epac1 or Epac2 mRNA affects SMC migration. We found that transfection of both siRNAs for Epac1 or Epac2 significantly decreased the expression of Epac1 (31–40%) or Epac2 (48–54%), respectively (Fig. 4, A and B). It should be noted that the siRNAs for Epac1 did not change the expression of Epac2 mRNA and vice versa. An siRNA for Epac1 significantly decreased serum-mediated SMC migration (~72%) (Fig. 4C), whereas that for Epac2 did not.

**Epac-enhanced Cytoskeletal Organization and Focal Adhesion in DA SMCs**—Since cytoskeletal remodeling plays an important role in cell motility (11), it is important to consider the effect of Epac on cytoskeletal organization and focal adhesion. We found that changes in cytoskeletal organization after Epac activation were quite different from those after PKA activation (Fig. 5). O-Me-cAMP induced a dramatic change in morphology within 60 min. Actin stress fibers were well organized without branched extensions, and focal adhesion detected by paxillin was more widely distributed not only in the edge of the cell but also on actin filaments as fibrous structures, consistent with a recent report showing co-localization of paxillin on actin stress fibers in an other cell (12). In the presence of pCPT-cAMP, paxillin staining was localized around the nucleus. When cells were simultaneously stimulated by O-Me-cAMP and pCPT-cAMP, the cell surface area was normalized, although the staining pattern of actin filaments and paxillin in these cells was different from control cells and O-Me-cAMP- or pCPT-cAMP-stimulated cells.

Interestingly, in the presence of ONO-AE1–329, DA SMCs displayed two distinct types of morphology (Fig. 6), which were...
different from the morphology of the cells stimulated simultaneously by O-Me-cAMP and pCPT-cAMP. PGE₂ stimulation showed a similar result with ONO-AE1–329 (data not shown). In one type of DA SMC (P-type, indicated in Fig. 6 as a white arrow), the morphology was similar to that of PKA-stimulated cells, and in the other type (E-type, indicated in Fig. 6 as an arrowhead), the morphology of E-type DA SMCs could look either like control cells or like Epac-stimulated cells. In 146–190 cells examined, the ratio of P-type to E-type cells was 1: 0.97, 1: 0.74, and 1: 0.92 at the 10⁻⁸, 10⁻⁷, and 10⁻⁶ M concentrations of ONO-AE1–329, respectively. Cell surface area was greater in E-type cells than in P-type cells. A significant increase in cell surface area was observed in E-type cells in accordance with an increase in a concentration of ONO-AE1–329, but in P-type cells, there was no change in surface area. The expression of paxillin was more widely distributed throughout the E-type cells in accordance with an increase in a concentration of ONO-AE1–329.

Epac Inhibited Cell Proliferation and Did Not Promote HA Production in DA SMCs—Our recent study showed that chronic PGE₂-cAMP-PKA stimulation increased HA production, resulting in the promotion of DA SMC migration and proliferation and thus ICF (3). It is now important to examine whether Epac regulates proliferation and HA production in DA SMCs. We found that O-Me-cAMP inhibited proliferation of rat DA SMCs (Fig. 7A).

We also found that O-Me-cAMP did not change HA production in rat DA SMCs (Fig. 7B). These data indicated that Epac-promoted migration was independent of SMC proliferation and HA production.

Epac1, but Not Epac2, Promoted ICF in the Rat DA —Finally, we investigated whether Epac1 and Epac2 indeed promoted ICF in the rat DA. Adenovirus-mediated Epac1 gene transfer induced prominent ICF in the rat DA explants, when compared with those with GFP gene transfer (Fig. 8A). However, Epac2 gene transfer did not. ICF, as determined by the ratio between neointimal area and medial area, became significantly greater in the Epac1-overexpressed DA, increasing by 1.98-fold (Fig. 8B), which suggests that Epac1 plays an important role in promoting neointimal thickening. Consistent with the findings in the previous section, both Epac1 and Epac2 overexpression did not increase the number of Ki67 positive cells and deposition of HA in DA explants (supplemental Fig. 2). Immunohistochemistry and quantitative RT-PCR analysis confirmed that the adenovirus-mediated Epac gene transfer indeed increased the expression of Epac in exo vivo DA (supplemental Fig. 3).
Epac Promotes Neointima in Ductus Arteriosus

A

GFP

Epac1

Epac2

B

Intimal Thickness (% of SM layer area)

GFP

Epac1

Epac2

FIGURE 8. Adenovirus-mediated Epac1 gene transfer promoted neointimal cushion formation in the rat DA explants. A, representative images of organ culture of immature rat DA overexpressed with GFP, Epac1, or Epac2. Scale bars, 100 μm. B, the ratio of the thickness of intimal cushion to the medial thickness of smooth muscle layer (SM layer). n = 3–4. The values are expressed as mean ± S.E. * indicates p < 0.05. ns, not significant.

DISCUSSION

The present study demonstrated that Epac promoted SMC migration and thus ICF in the rat DA. Since chronic cAMP-PKA stimulation promotes ICF in the rat DA (3), both EP4-cAMP downstream targets, Epac and PKA, induced ICF in the DA. However, their molecular mechanisms are different. PKA promotes ICF through accumulation of HA production that accelerates migration and proliferation of DA SMCs. Therefore, chronic stimulation (>48 h) of PKA is required to accumulate HA production (3). Notably, the present study demonstrated that acute stimulation (4 h) of PKA inhibited DA SMC migration, which is consistent with the previous studies on other vascular SMCs (9, 10). Epac, on the other hand, promoted SMC migration within 4 h. Furthermore, Epac-promoted SMC migration was inhibited by PKA stimulation. Such an opposing effect of Epac and PKA has been reported in other types of cells (13, 14).

Since Epac did not increase HA production in DA SMCs, Epac-promoted SMC migration was independent of HA. Besides, Epac stimulates Rap1, which is known to increase integrin-mediated cell adhesion to fibronectin (15). Along this line, we found that Epac activated Rap1 (Fig. 2) and focal adhesion (Fig. 5) in DA SMCs. Since fibronectin plays a critical role in promoting migration of DA SMCs (16), our data suggested that a Rap1-mediated increase in cell adhesion to fibronectin is responsible, at least in part, for Epac-promoted SMC migration.

Until now, the functional differences between Epac1 and Epac2 have not been fully understood. The present study demonstrated that Epac1 promoted SMC migration and thus ICF stronger than Epac2, although the relative expression levels of endogenous Epac2 mRNA were approximately two times higher than those of Epac1 mRNA during a perinatal period when ICF develops. Although Epac2 overexpression significantly increased SMC migration, it did not promote ICF in the DA explants. We used LacZ overexpression for SMC migration and GFP overexpression for organ culture as control experiments, since it has been demonstrated that GFP increased cyclooxygenase-2 expression and increased PGE2 release in some cell types (17). Therefore, one may assume that GFP-mediated PGE2 release promoted ICF in the DA explants, which may result in no difference between GFP and Epac2 overexpression in the present study. Anyway, using the same titer of adenoviral vectors, Epac1 overexpression promoted ICF stronger than Epac2 or GFP overexpression. Our previous study demonstrated that Epac1 and Epac2 mRNAs were up-regulated in isoproterenol-induced left ventricular hypertrophy, whereas only Epac1 was increased in pressure-overload-induced hypertrophy (6). In addition, we have recently found that Epac1, but not Epac2, is up-regulated in the model of balloon-induced vascular injury.4 Since the physiological process of ICF in the DA closely resembles the pathological process of ICF caused by vascular injury or atherosclerosis in adult arteries (18), Epac1 may play a central role in promoting ICF.

Another important observation in this study is that DA SMCs displayed two distinct types of morphology when stimulated with an EP4 agonist, ONO-AE1–329 or PGE2. P-type cells were easily distinguished since they closely resembled PKA-stimulated cells in that they showed disassembly of actin stress fibers and long branching extensions. Since the morphology of E-type cells resembled Epac-stimulated cells or control cells, it was difficult to distinguish these three cell types from one another. It should be noted that Epac activation is required to obtain a concentration of an agonist of a G protein-coupled receptor that is higher than the concentration of PKA (4). Therefore, we assumed that the ratio of Epac-stimulated cells should be increased in accordance with increases in the concentration of ONO-AE1–329 in the rat DA SMCs. Accordingly, the cell surface area and cell adhesion of E-type cells were increased in the presence of a high concentration of ONO-AE1–329 (10^-6 M) in the rat DA SMCs. Interestingly, when cells were simultaneously stimulated by both PKA and Epac agonists, the cell surface area of cells was comparable with control cells, and the appearance of the cells was homogeneously similar among cells. They did not resemble P-type or E-type cells. Thus, simultaneous stimulation of PKA and Epac was different from stimulation of EP4. These data suggested that when cells are activated by EP4 stimulation, one pathway, PKA or Epac, may be dominantly activated in each cell. The molecular mechanism by which cells respond differently to PGE2-EP4 stimulation remains unknown. Further study is apparently required to identify what kind of switch makes these cells follow diverse pathways.

In conclusion, the present study demonstrated that Epac, which is up-regulated during the perinatal period, had an acute promoting effect on SMC migration and thus on ICF in the DA.

The PGE2-EP4 signal activates the diverse cAMP pathways, both Epac and PKA, which synergistically promote ICF in the DA in two distinct ways, namely acute (Epac1-mediated) and chronic (PKA-mediated) (Fig. 9). Although further in-depth investigation will be required to understand the molecular mechanism of Epac-mediated ICF in the DA, our results imply that selective activation of Epac may serve as an alternative therapeutic strategy for patients with patent DA.

**REFERENCES**