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Prostaglandin E_2-activated Epac Promotes Neointimal Formation of the Rat Ductus Arteriosus by a Process Distinct from That of cAMP-dependent Protein Kinase A*[S]

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We have demonstrated that chronic stimulation of the prostaglandin E_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-pCPT-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_2 (PGE_2)[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_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_2 stimulation has another essential effect on DA development, namely intimal cushion formation (ICF) (3). Briefly, via EP4, a predominant PGE_2 receptor in the DA, the PGE_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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[3] The abbreviations used are: PGE_2, prostaglandin E_2; PKA, cAMP-dependent protein kinase A; DA, ductus arteriosus; PKI-(14-22)-amide, PKA inhibitor-(14-22)-amide; *O*-Me-cAMP, 8-pCPT-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.

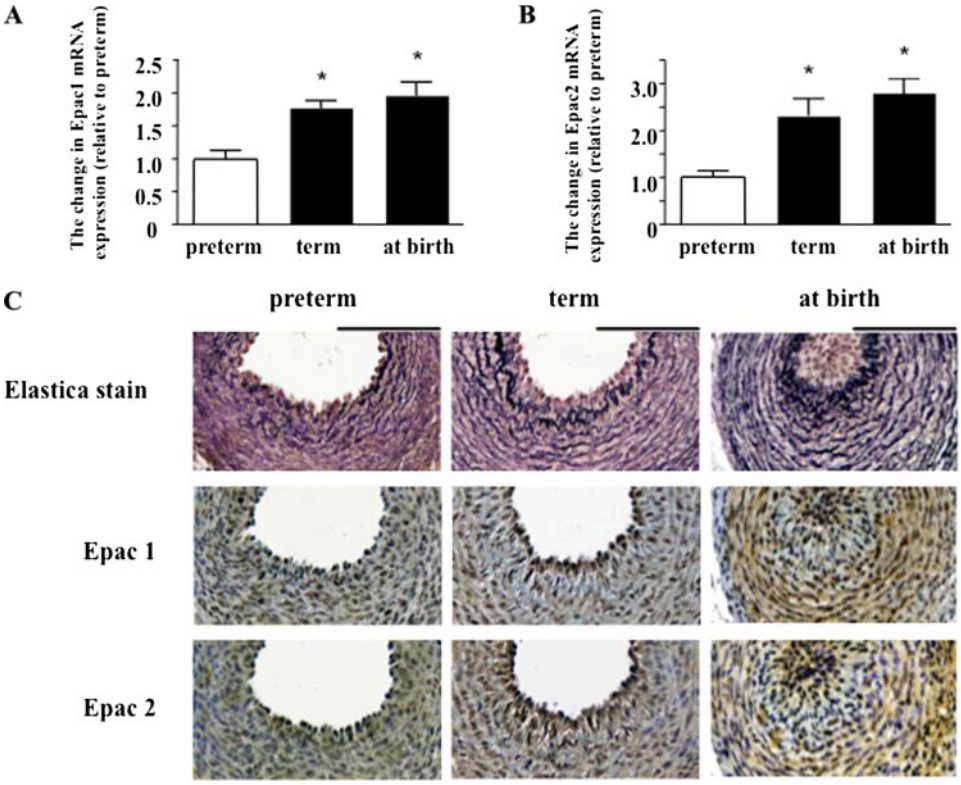

FIGURE 1. **Epac expression in the rat DA.** *A* and *B*, the levels of *Epac1* (*A*) and *Epac2* (*B*) mRNAs in the rat DA during development ($n = 7$). *C*, immunohistological analysis of Epac1 (*middle line*) and Epac2 (*bottom line*) proteins in the rat DA on the 19th day of gestation (*preterm*) (*left panel*), on the 21st day of gestation (*term*) (*middle panel*), and at birth (neonate) (*right panel*). Epac1 or Epac2 was detected in a brown color. Dark blue stain was counterstained with Mayer's hematoxylin. *Scale bars*, 100 μm. The values are expressed as mean ± S.E. * indicates $p < 0.05$ to control.

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 tga tgc acg gat ga-3′ (forward) and 5′-aaa ctg ctg caa aag cac ct-3 (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 phalloidin antibody (Invitrogen) and paxillin antibody (BD Biosciences) for 16 h at 4 °C. Some specimens with a primary antibody were prepared without

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% confluency, 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′-uug ugc aca ugc ucu gug g(tg)-3′ and 5′-gag aug acg gua cag gag c(tt)-3′. The antisense siRNA sequences targeting *Epac2* were 5′-uag auu guc aau gaa ugu c(tt)-3′ and 5′-uaa aug acu aca uuc acg 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 using a measurement of [^{3}H]thymidine incorporation as described previously (5). 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 [^{3}H]methyl thymidine (specific activity 5 Ci/mmol/liter; Amersham Biosciences International) for 4 h at 37 °C. Data obtained from triplicate wells were averaged.

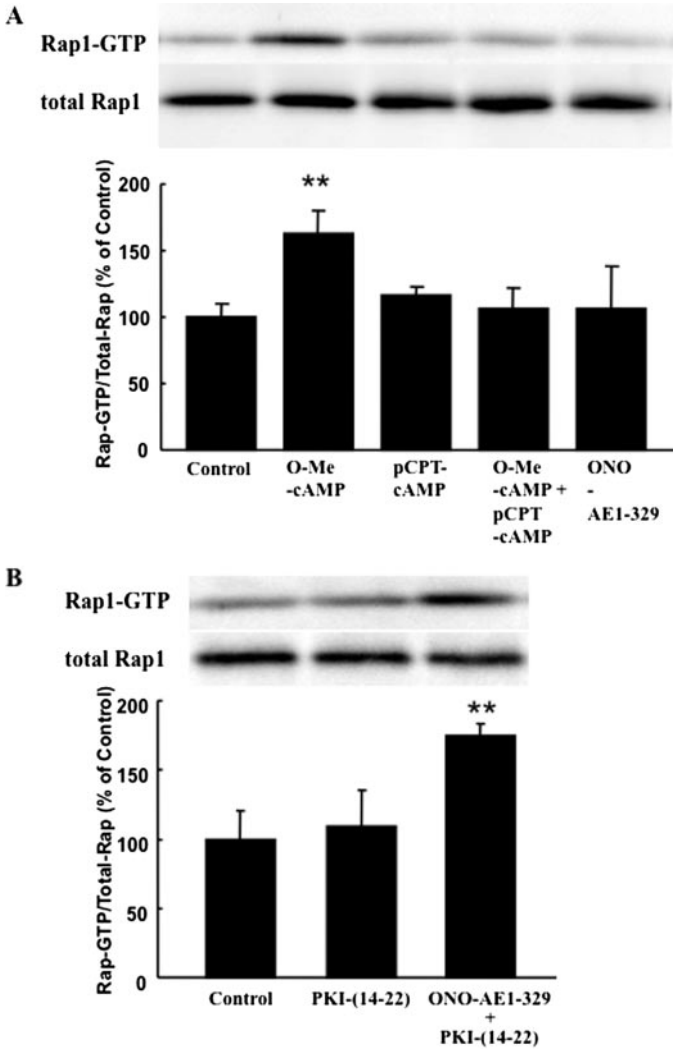

FIGURE 2. **Rap1 was activated by Epac or EP4 stimulation with PKA inhibition.** *A*, Rap1 activity assay revealed that *O*-Me-cAMP (30 μM), a cAMP analog selective to Epac (8), strongly increased Rap1 activity in DA SMCs, whereas pCPT-cAMP (500 μM), a cAMP analog selective to PKA or a specific EP4 agonist, 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.

Organ Culture—DA organ culture was performed as described previously (3). Briefly, fetal arteries removed on the 19th day of gestation, including the DA, were infected for 2 h with 1.2×10^7 plaque-forming unit/ml of the *Epac1*, *Epac2*, or *GFP* adenovirus in 0.5% fetal calf serum containing Dulbecco's modified Eagle's medium in humidified 5% CO_2 and 95% ambient mixed air at 37 °C. After infection, the segments were cultured for up to 2 days, fixed in 10% buffered formalin, and embedded in paraffin. Morphometric analyses were performed using Win Roof version 5.0 software (Mitani Corp., Tokyo, Japan). Intimal cushion formation was defined as ((neointimal area)/(medial area)) × 100%.

Statistical Analysis—Data are the means ± S.E. of independent experiments. Statistical analysis was performed between two groups by unpaired Student's *t* test or unpaired *t* test with Welch correction and between multiple groups by one-way

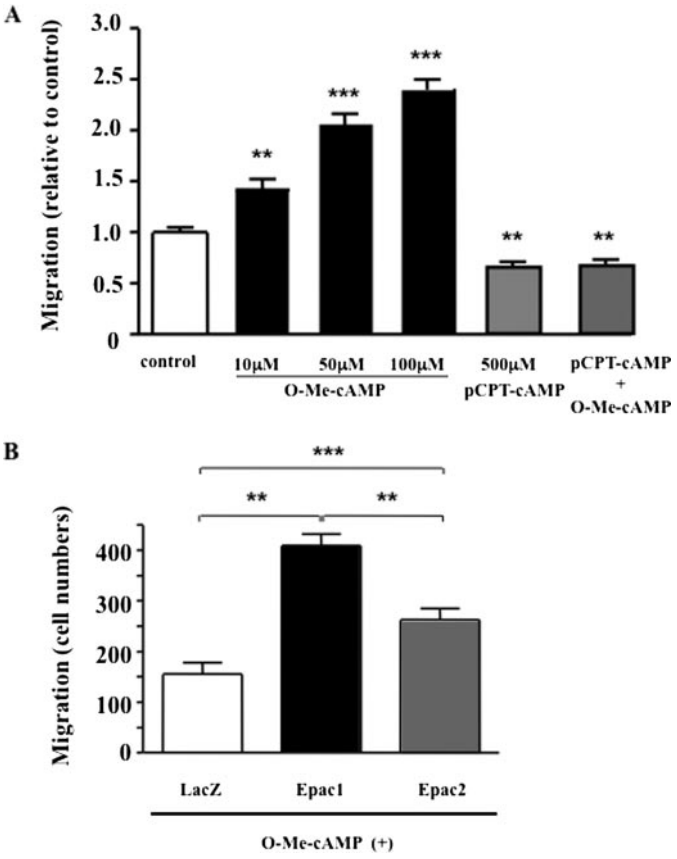

FIGURE 3. **Epac promoted DA SMC migration.** *A*, the effect of *O*-Me-cAMP and pCPT-cAMP on rat DA SMC migration. *O*-Me-cAMP increased SMC migration in a dose-dependent manner. $n = 3–4$. The values are expressed as mean ± S.E. ** and *** indicate $p < 0.01$ and $p < 0.001$ to control, respectively. *B*, the effect of adenovirus-mediated *Epac1* or *Epac2* gene transfer on *O*-Me-cAMP-promoted migration. Overexpression of *Epac1* and *Epac2* significantly increased SMC migration. *LacZ* gene transfer was used as control. $n = 4$. The values are expressed as mean ± S.E. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively.

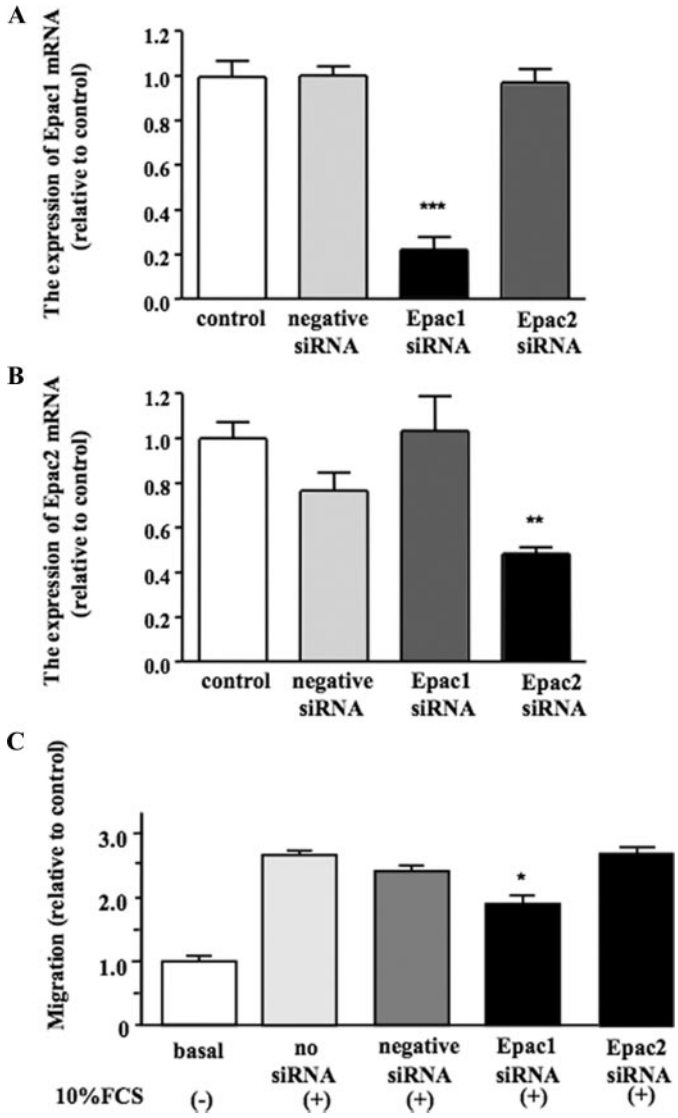

FIGURE 4. **siRNA for Epac1 decreased serum-mediated migration of DA SMCs.** *A*, the effect of transfection of siRNAs on the expression of *Epac1* mRNA. The values are expressed as mean ± S.E. *** indicates $p < 0.001$ to control and negative siRNA. *B*, the effect of transfection of siRNAs on the expression of *Epac2* mRNA. The values are expressed as mean ± S.E. ** indicates $p < 0.01$ to control and negative siRNA. *C*, the effect of transfection of siRNAs on serum-mediated SMC migration. $n = 6–7$. The values are expressed as mean ± S.E. * indicates $p < 0.05$ to control and negative siRNA. *FCS*, fetal calf serum.

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. 1*C*). 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. 2*A*). 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. 2*A*). 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. 2*B*). These data indicated that EP4 activated not only cAMP-PKA but also cAMP-Epac pathway in DA SMCs.

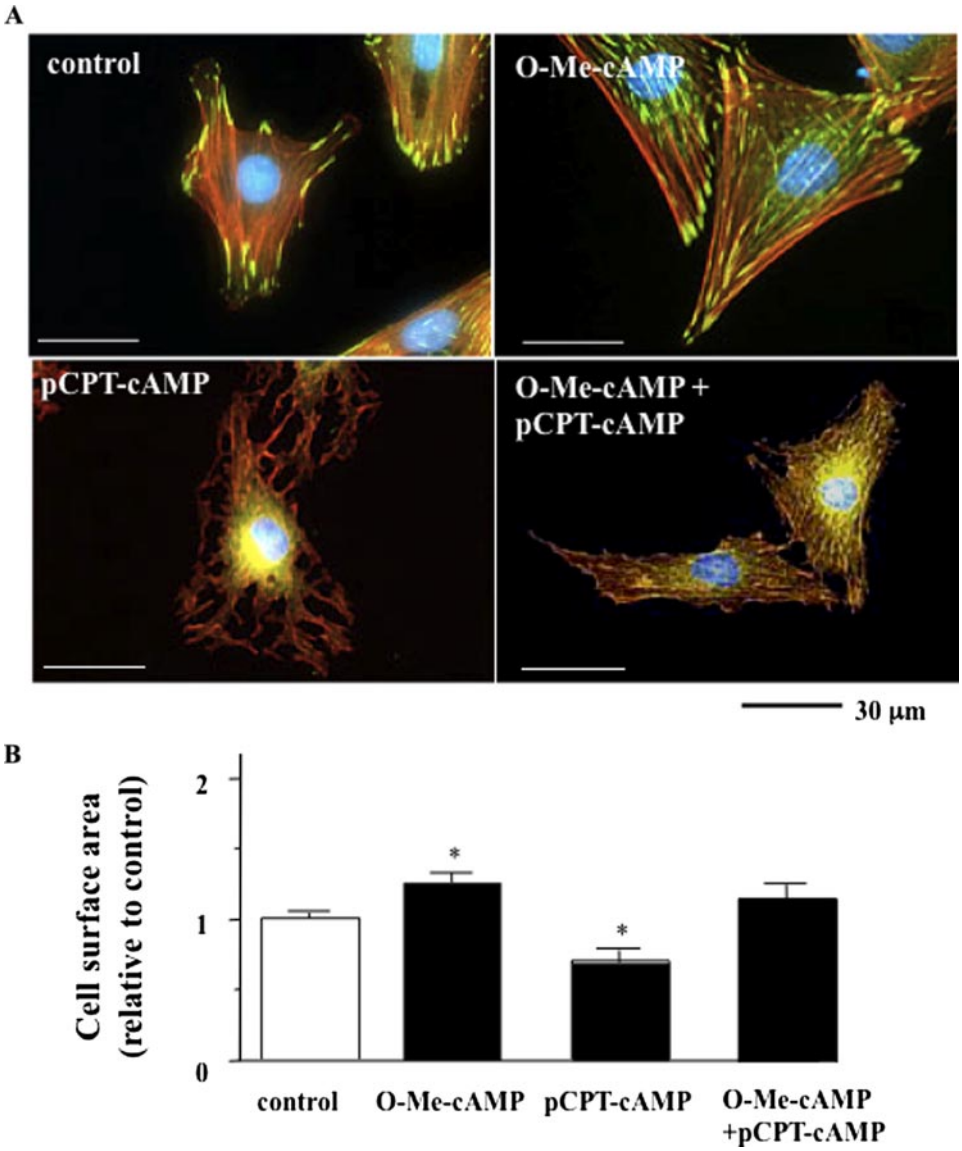

FIGURE 5. **Epac enhanced cytoskeletal organization and focal adhesion.** *A*, the effect of Epac or PKA activation on cytoskeletal organization and cell adhesion. In the presence of *O*-Me-cAMP, actin stress fibers (*red*) were well organized without branched extensions, and focal adhesion detected by paxillin (*green*) was increased. pCPT-cAMP, on the other hand, induced disassembly of actin stress fibers, and paxillin was localized in the perinuclear lesion. *Scale bars*, 100 μm. *B*, cell surface area was significantly increased by *O*-Me-cAMP and significantly decreased by pCPT-cAMP. The values are expressed as mean ± S.E. * indicates $p < 0.05$ to control.

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. 3*A*). On the contrary, acute stimulation (4 h) of pCPT-cAMP significantly inhibited DA SMC migration (Fig. 3*A*), 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. 3*B*). However, the effect of *Epac1* overexpression on cell migration was much stronger that 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. 4*C*), 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, and cell shape was widely expanded, mimicking epithelial cells without branched extensions. On the other hand, pCPT-cAMP induced disassembly of actin stress fibers and long branching extensions. Cell surface area was significantly increased with Epac activation and significantly decreased with PKA activation (Fig. 5B). The expression of paxillin was mainly located in the edge of the control cells. In the presence of *O*-Me-cAMP, paxillin was more widely distributed not only in the edge of the cells 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

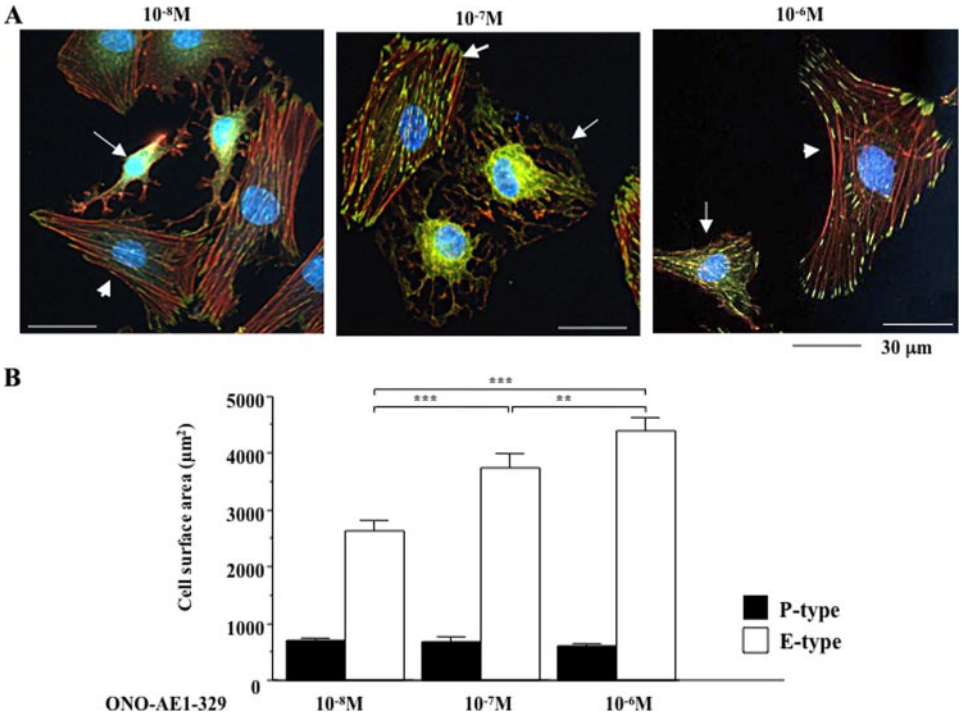

FIGURE 6. **The change in cell morphology due to EP4 stimulation.** *A*, DA SMCs were incubated with different concentrations of ONO-AE1–329 for 1 h and then subjected to immunostaining using phalloidin (*red*) and paxillin (*green*) antibodies. A *white arrow* and an *arrowhead* indicate P-type and E-type SMCs, respectively. *Scale bars*, 30 μm. *B*, changes in cell surface area of E-type and P-type SMCs in the presence of ONO-AE1–329. $n = 50$. The values are expressed as mean ± S.E. ** and *** indicate $p < 0.01$ and $p < 0.001$.

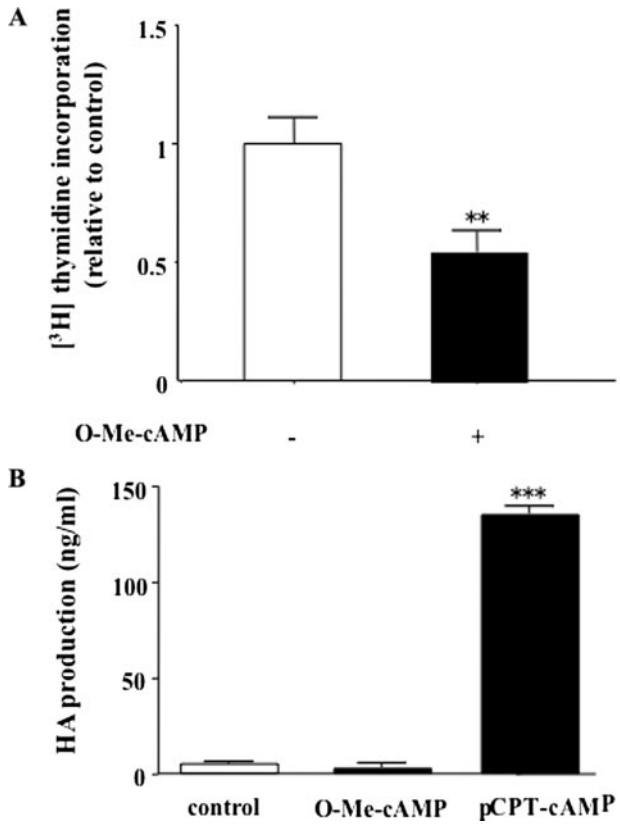

FIGURE 7. **Epac did not promote cell proliferation and HA production in DA SMCs.** *A*, the effect of *O*-Me-cAMP on proliferation of rat DA SMCs. $n = 3–4$. *B*, the effect of *O*-Me-cAMP and pCPT-cAMP on HA production in rat DA SMCSs. $n = 4$. The values are expressed as mean ± S.E. ** and *** indicate $p < 0.01$ and $p < 0.001$ to control, respectively.

different from the morphology of the cells stimulated simultaneously by *O*-Me-cAMP and pCPT-cAMP. PGE_2 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 PGE_2-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. 7*A*).

We also found that *O*-Me-cAMP did not change HA production in rat DA SMCs (Fig. 7*B*). 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. 8*A*). 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. 8*B*), 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).

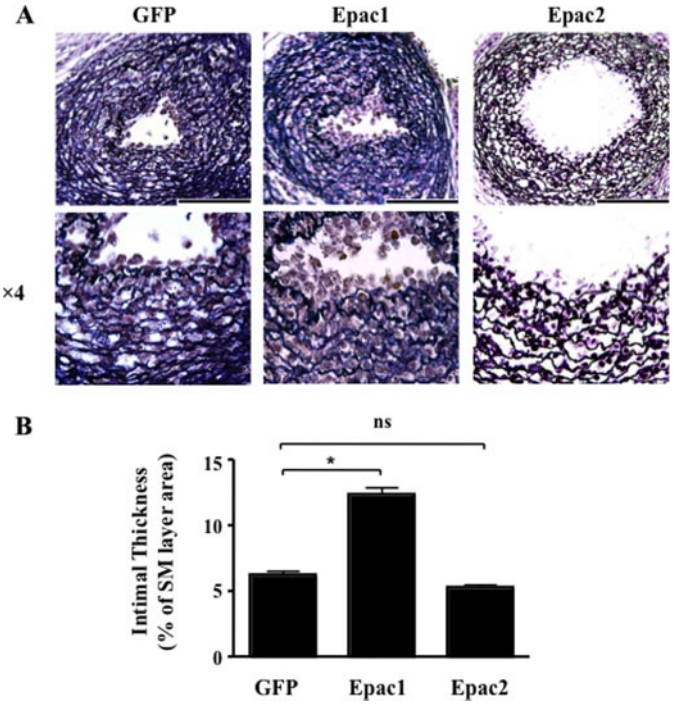

FIGURE 8. **Adenovirus-mediated *Epac1* gene transfer promoted neointimal cushion formation in the rat DA explants.** *A*, representative images of organ culture of immature rat DA overexpressed with *GFP*, *Epac1*, or *Epac2*. *Scale bars*, 100 μm. *B*, the ratio of the thickness of intimal cushion to the medial thickness of smooth muscle layer (*SM layer*). $n = 3–4$. The values are expressed as mean $\pm$ 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 rat 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 PGE_2 release in some cell types (17). Therefore, one may assume that GFP-mediated PGE_2 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 PGE_2. 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 PGE_2-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.

[4] U. Yokoyama, S. Minamisawa, T. Akalke, M. Sata, and Y. Ishikawa, unpublished data.

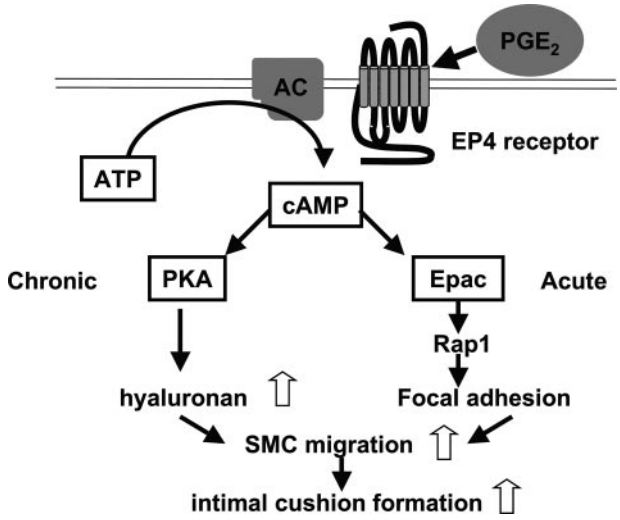

FIGURE 9. **A scheme of the diverse cAMP signal pathways.** Both Epac and PKA synergistically promoted intimal cushion formation in the DA, but they work in two distinct ways: Epac1-mediated (acute) and PKA-mediated (chronic) promotion. AC, adenylyl cyclase.

The PGE_2-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.

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