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RESEARCH ARTICLE

Mechanisms regulating cilia growth and cilia function in endothelial cells

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Abstract The primary cilium is an important sensory organelle present in most mammalian cells. Our current studies aim at examining intracellular molecules that regulate cilia length and/or cilia function in vitro and ex vivo. For the first time, we show that intracellular cAMP and cAMP-dependent protein kinase (PKA) regulate both cilia length and function in vascular endothelial cells. Although calcium-dependent protein kinase modulates cilia length, it does not play a significant role in cilia function. Cilia length regulation also involves mitogen-activated protein kinase (MAPK), protein phosphatase-1 (PP-1), and cofilin. Furthermore, cofilin regulates cilia length through actin rearrangement. Overall, our study suggests that the molecular interactions between cilia function and length can be independent of one another. Although PKA regulates both cilia length and function, changes in cilia length by MAPK, PP-1, or cofilin do not have a direct correlation to changes in cilia function. We propose that cilia length and function are regulated by distinct, yet complex intertwined signaling pathways.

Keywords Calcium signaling · Cardiovascular homeostasis · Ciliopathy · Fluid-shear stress · Intraflagellar transport · Mechanosensory cilium

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Introduction

Primary cilia are solitary organelles that extend from the basal body of the apical surface into the extra cellular matrix of most eukaryotic cells. Their unique position and structure bestow an added advantage of vastly increasing the area of contact with the cell exterior [1]. A primary cilium houses a large number of proteins. It has been suggested that a cilium has five distinct domains [2]. One of these domains includes the soluble compartment, which is also called the matrix compartment or cilioplasm. The cilioplasm is composed of fluid material to support various signaling proteins.

Cilia length and cilia function play a major role in maintaining a healthy cellular state. Studies on primary cilia in the renal epithelial system show that the length of the cilia is highly regulated, with the cilia being longer in larger lumen and shorter in smaller lumen [3]. Many studies have also found that cilia length can be regulated under different physiological conditions [4, 5]. Since the length of the cilia is very important in many organs involving fluid flow, such as the kidneys, pancreas, liver and many others, it is important to determine the mechanisms involved in cilia length and function. Most important, the relationship between cilia length and function remains unknown.

In the current study, we have used pharmacological agents to modify the level of intracellular cAMP and activity of various protein kinases and phosphatase. We identify molecular pathways that regulate cilia length. Interestingly, this regulation may also involve actin rearrangement. Our functional assay on cilia further indicates that mechanosensory cilia function may not always coincide with changes in the cilia length. Overall, our studies offer more detailed insight into regulation of cilia structure and function.

Materials and methods

Animal and cell culture

The Institutional Biosafety Committee of The University of Toledo approved the use of endothelial cells and other biohazard reagents. The University of Toledo Animal Care and Use Committee approved the use of animal tissues. In our studies, we used vascular endothelial cells that were previously described and characterized for various surface and intracellular markers [6, 7].

Cilia analysis and measurement

To measure cilia length, cells or femoral arteries were observed with fluorescence and scanning electron microscopes. For fluorescence analysis, cells and tissue were fixed with 4% paraformaldehyde in 2% sucrose solution for 10 min at room temperature. Acetylated α-tubulin was used as a ciliary marker and to measure cilia length. Antibodies used included acetylated α-tubulin (anti-mouse, Sigma clone 6-11B-1; 1:10,000 dilution), FITC (anti-mouse, 1:500, Vector Laboratory), Texas Red®-X phalloidin (anti-mouse, 1:100, Invitrogen) and DAPI (Vector Laboratory). The cover slip was then mounted on the microscope slide with mounting media containing DAPI. Cilia images were observed with an inverted Nikon Ti-U microscope and analyzed three dimensionally with Metamorph 7.0.

For the scanning electron micrograph, cells or femoral arteries were fixed with 2.5% paraformaldehyde/glutaraldehyde in sodium cacodylate buffer for 1 h at room temperature. In case of femoral artery, after fixing and drying the piece for 24 h, we made very fine cross sections of the artery ($\sim$1 mm) as such that the lumen would always be exposed for analysis. The samples were chemically dried using an initial 2-h incubation in 50% HMDS-ethyl alcohol mixture, followed by two half-hour incubations in 100% HMDS. Micrographs were obtained and analyzed using a Hitachi HD-2300 scanning electron microscope.

cAMP measurement

The measurement of cAMP was carried out according to the cAMP EIA kit with acetylation step (Cayman Chemicals). In every measurement, a standard curve of cAMP was generated with a typical correlation coefficient of 0.992 ± 0.001. The standards and samples were read at a wavelength of 410 nm in duplicates and triplicates, respectively. The total cAMP was normalized with total protein content determined by a standard Bradford assay.

Cytosolic calcium measurement

For cilia function analysis, we used a similar protocol and setup as previously described [6, 7]. Briefly, cells were loaded with 5 μM Fura2-AM (Invitrogen) for 30 min at 39°C. Basal calcium was first determined for about a minute. Fluid flow at optimal shear stress was used to monitor changes in cytosolic calcium every 4 s. Changes in cytosolic calcium were monitored and recorded using a Nikon TE2000 microscope and Metafluor software. At the end of the experiment, the minimum fluorescence was determined by treating the cells with 2 mM EGTA and 10 μM ionomycin. After achieving the minimum signal, the maximum fluorescence was obtained by treating the cells with excess calcium (10 mM). All fluorescence measurements were corrected for auto-fluorescence.

Protein analysis

Measurement of protein was performed using a standard Western-blot analysis. Each well was loaded with 150 μg of protein. The membranes were initially blocked for 2 h with 5% milk in TBS containing 1% Tween. All the antibodies were diluted in 1% milk in TBST solution. The following antibodies were used in our analysis: anti-phospho ERK (anti-rabbit, Cell Signaling, 1:1,000 dilution, overnight at 4°C), p-cofilin (anti-rabbit, Cell Signaling, 1:100, overnight at 4°C), anti-ERK (anti-rabbit, Cell Signaling, 1:1,000 dilution, overnight at 4°C), and anti-α-tubulin (anti-rabbit, Abcam, 1:5,000 dilution, overnight at 4°C).

Pharmacological treatment

The following pharmacological agents and their concentrations were used on cells and tissues: cAMP analog (8pCPT-cAMP; 10 μmol/l), PKA activator (forskolin; 10 μmol/l), PKC activator (phorbol myristate acetate; 0.5 μmol/l), PKC inhibitor (bisindolylmaleimide XI hydrochloride; 0.5 μmol/l), and MAPK inhibitor (PD98059, 10 μmol/l). These concentrations were titrated to have optimal effects in our system. High-grade-quality pharmacological agents were selected and purchased from Sigma.

Agents were added after the cells were differentiated at 39°C to avoid any potential effect on cell growth. For femoral arteries, isolated tissues were briefly cleaned and rinsed with phosphate buffer containing glucose and calcium. Fresh buffer (2 ml) and the drug were added, and the samples were incubated at 39°C for the desired durations. Drugs were incubated with the samples for 4 or 16 h. The data for 4 h are not shown because of the inconsistency due to a non-optimal condition. Extreme caution was taken

when adding the drugs. Drugs were diluted to different concentrations so that the exact same volume of the drug solution was added to the samples in an effort to maintain identical volume.

Statistical analysis

To examine effects of cAMP on cilia function, we determined the biological function by studying a typical logarithmic scale dose–response curve ($\log_e(x)$) [8, 9]. Likewise, we used the same mathematical function for the cAMP measurement, which was also suggested by the manufacturer. The following equation was used to obtain the cAMP standard curve: $y = \log_e(x) + c$; where y is $\log_{10}$ of signal intensity, x represents cAMP concentration, and c denotes proportional coefficient.

Unless otherwise indicated, analysis was done after 16 h of treatment with pharmacological agents. All quantifiable experimental values are expressed as mean ± SEM, and values of $p < 0.05$ are considered significant. All comparisons between two groups were performed with Student's t test. Comparisons of three or more groups were done using ANOVA, followed with Tukey's posttest. Data analysis was performed using SigmaPlot software version 11.

Results

Cilia length is regulated by various protein kinases in mouse endothelial cells and tissues

Previous studies have shown that intracellular cAMP regulates cilia length in cultured mono-layered epithelial cells [10, 11]. Our present studies also indicate that cAMP can increase cilia length in mouse endothelia of mono-layered cells in vitro and femoral arteries ex vivo (Fig. 1). We next utilized various pharmacological agents to examine the

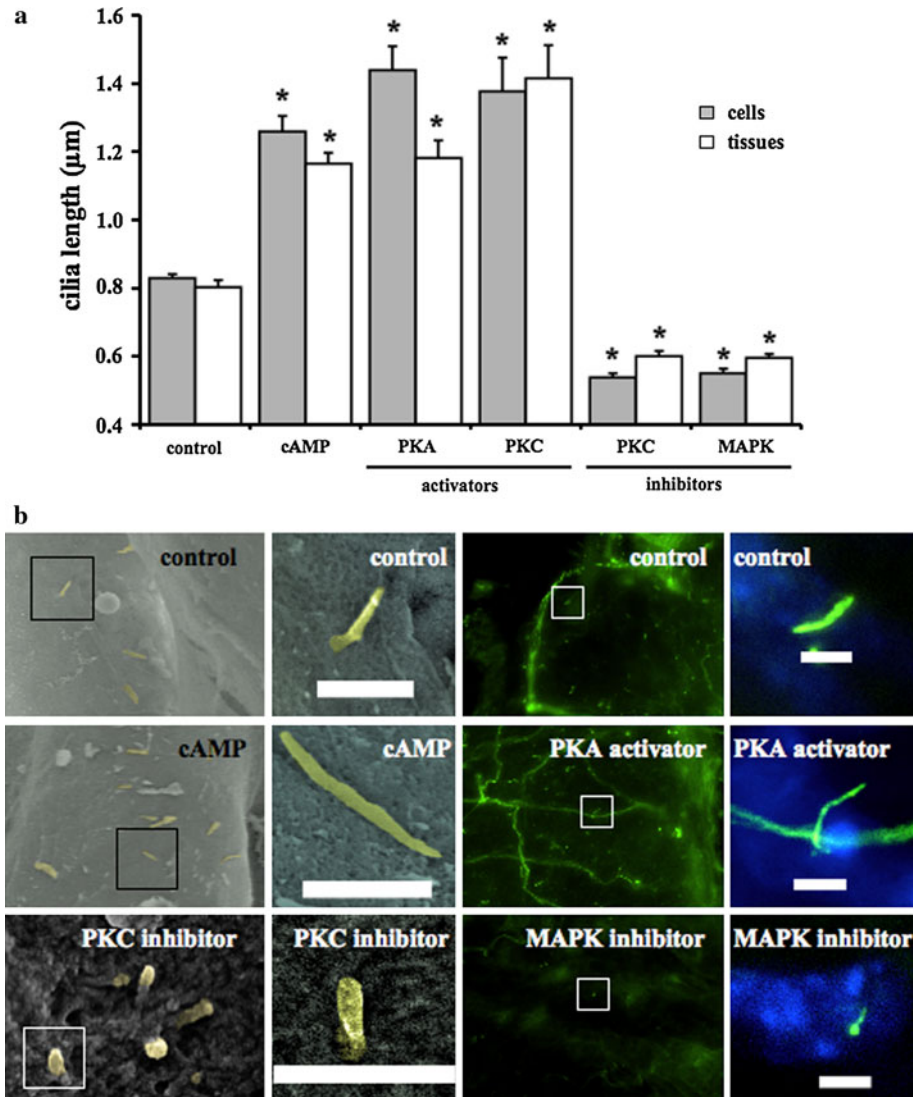

Fig. 1 Cilia length can be modulated with pharmacological agents. **a** Length of primary cilia in mono-layered cells and femoral arteries was measured before (control) and after treatment with 8-pCPT-cAMP (cAMP, 10 μmol/l), forskolin (PKA activator, 10 μmol/l), PMA (PKC activator, 0.5 μmol/l), bisindolylmaleimide (PKC inhibitor, 0.5 μmol/l), or PD98059 (MAPK inhibitor, 10 μmol/l) for 16 h. The cAMP analog and activators significantly increased cilia length, while the inhibitors significantly decreased cilia length. **b** Representative scanning electron and immunofluorescence images from femoral arteries are shown. Acetylated-α-tubulin was used to identify the primary cilia. Boxes indicates a greater magnification from the field of view. *Bar* 1 μm. $n = 3$ independent experiments; over 120 cilia were measured in cultured cells, and about 50 cilia were measured in femoral arteries. *$p < 0.05$ compared to control group

roles of other kinases. Activating cAMP-dependent protein kinase (PKA) or calcium-dependent protein kinase (PKC) is sufficient to significantly increase cilia length in cultured and femoral endothelial cells. On the other hand, inhibiting PKC or mitogen-activated protein kinase (MAPK) significantly decreases cilia length. Our data indicate that endothelial cilia can be modulated by cAMP, PKA, PKC, and MAPK. This modulation is observed in both cultured cells and vascular tissues.

Cilia length is regulated by ERK and cofilin

To further understand the mechanism involved in cilia length regulation, we first examined the effects of the kinases' activities on intracellular cAMP level (Fig. 2a). PKA activation significantly increases intracellular cAMP level, indicating a possible synergistic pathway of cAMP and PKA in regulating cilia length. On the other hand, PKC activation does not increase cAMP level, although it increases cilia length. In addition, although PKC or MAPK inhibition decreases cilia length, neither has a significant effect on cAMP level compared to control. This suggests that PKC/MAPK is downstream or has a separate pathway from cAMP.

We next investigated this possibility by measuring ERK phosphorylation, as an indicator of MAPK activity. Because activation of MAPK can also promote cofilin dephosphorylation or activation [12, 13], we also examined cofilin phosphorylation level (Fig. 2b). Our data show that ERK phosphorylation is consistently and substantially increased by cAMP, PKA activation, or PKC activation at 15 and 30 min. Compared to control, PKC or MAPK inhibition has a minimal effect on ERK phosphorylation. Most interesting is that ERK phosphorylation within 15 min is inversely correlated with cofilin phosphorylation. In other words, ERK phosphorylation promotes activation of cofilin.

Cilia length is regulated by actin rearrangement and protein phosphatase-1

Cofilin is an actin-binding protein that regulates assembly and disassembly of cytoskeletal actin filament rearrangement [12, 13]. Thus, activation or dephosphorylation of cofilin induces actin reorganization. To examine this possibility in our endothelial cells, we analyzed actin cytoskeleton in the absence or presence of cAMP analog, PKA activator, PKC activator, PKC inhibitor or MAPK

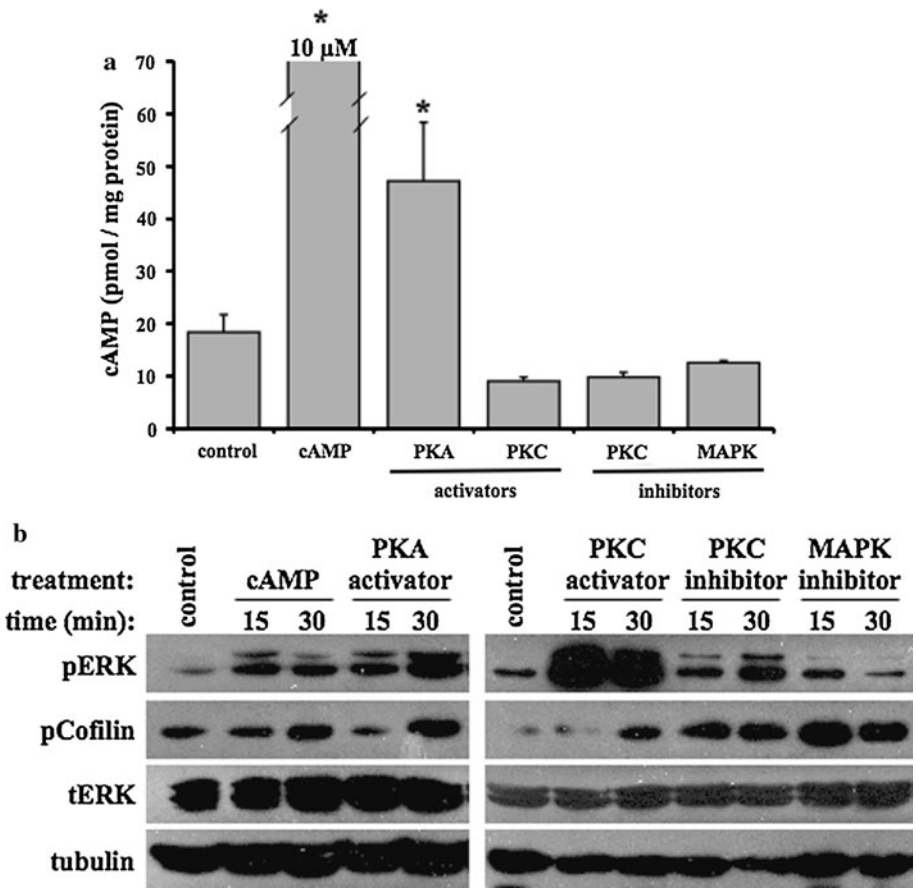

Fig. 2 Ciliary length modulation involves a combination of cAMP, pERK, and cofilin activation. **a** Intracellular cAMP was measured in untreated cells (control) and cells treated with 8-pCPT-cAMP (cAMP), forskolin (PKA activator), PMA (PKC activator), bisindolylmaleimide (PKC inhibitor), or PD98059 (MAPK inhibitor). **b** Western blots depicting phosphorylated ERK (pERK), phospho-cofilin (pCofilin), total ERK (tERK), and α-tubulin (tubulin) are shown in cells with corresponding treatments for 15 or 30 min. α-tubulin was used as a loading control. $n = 3$ independent experiments for cAMP measurement and two for Western blots. $^*p < 0.05$ compared to control group

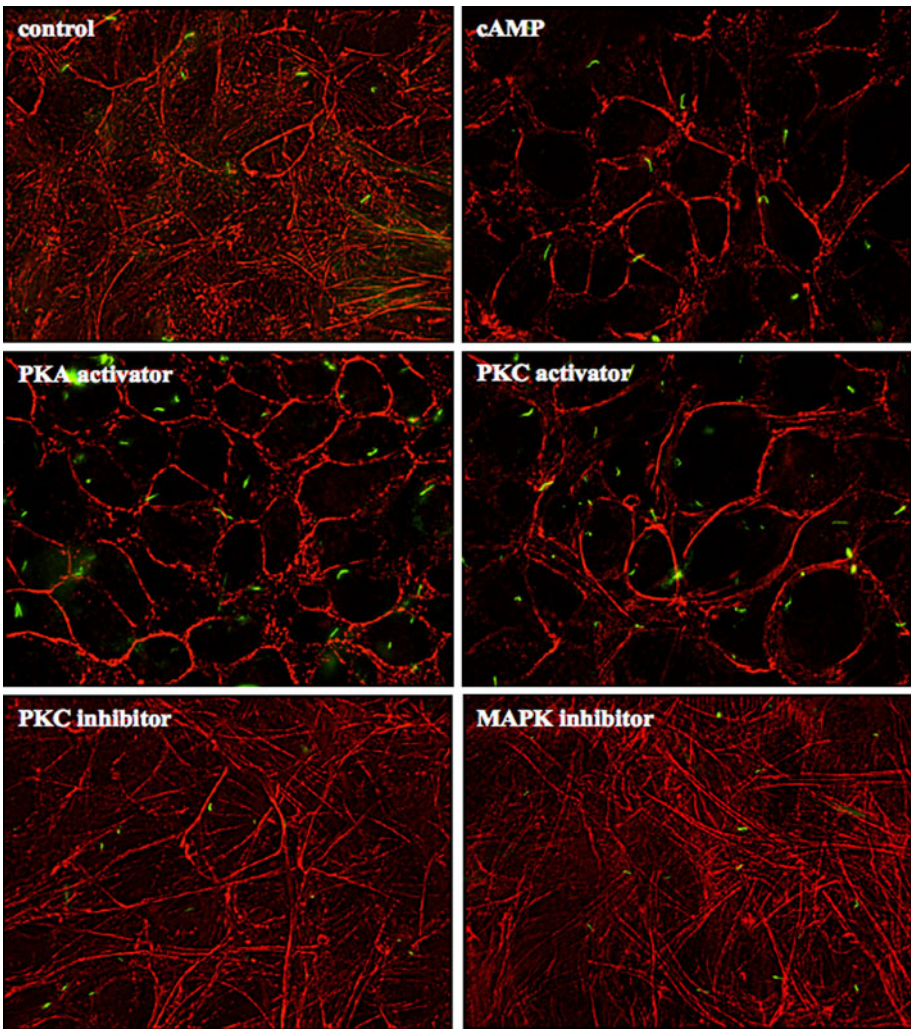

Fig. 3 Ciliary length modulation coincides with actin rearrangement. Representative images of primary cilia (*green*) and cytoskeletal actin filament (*red*) are shown. Actin filament was examined with phalloidin in untreated cells (control) and cells treated with 8-pCPT-cAMP (cAMP), forskolin (PKA activator), PMA (PKC activator), bisindolylmaleimide (PKC inhibitor), or PD98059 (MAPK inhibitor). Filamentous actin stress fibers are reorganized to form cortical actin in cells treated with cAMP, PKA activator, or PKC activator. On the other hand, cells treated with PKC inhibitor or MAPK inhibitor did not show apparent difference in actin stress fibers when compared to control. $n = 3$ independent experiments

inhibitor (Fig. 3). As predicted, a normal distribution of actin stress fiber is consistently restructured to cortical filamentous actin in the presence of cAMP analog, PKA activator, or PKC activator. On the other hand, cells treated with PKC or MAPK inhibitors exhibit similar actin stress fibers as those observed in the control.

Protein phosphatase-1 (PP-1) can dephosphorylate and thereby activate cofilin [14]. It is therefore expected that inhibition of PP-1 activity would block cAMP-, PKA- or PKC-induced filamentous actin rearrangement (Fig. 4a). This further suggests that cofilin works downstream to PKA and MAPK. We hypothesize that if cofilin is involved in regulating cilia length, inhibition of PP-1 will result in a decrease in cilia length. Supporting our hypothesis, co-incubation of PP-1 inhibitor with cAMP analog, PKA activator or PKC activator significantly decreases cilia length (Fig. 4b). Most surprising is that inhibition of PP-1 basal activity is sufficient to shorten cilia length, as indicated in the control group. We propose that PP-1 and cofilin-induced actin rearrangement play an important role in cilia length maintenance.

Cilia function is regulated by cAMP and PKA

One of the many roles of primary cilia is to function as a mechanical sensor [15, 16]. To understand if cilia function is also regulated by cAMP, PKA, PKC, and/or MAPK, we performed mechanical fluid-shear experiments to examine cilia function (Fig. 5a). To enable us to assay relative functions of cilia, we calculated the amount of total cytosolic calcium increase in response to fluid-shear stress. Total changes in cytosolic calcium are determined by the area under the calcium-time curve (area under the curve). Compared to the control group, only cells treated with cAMP analog or PKA activator show a significant increase in cilia function (Fig. 5b). PKC and MAPK do not play a significant role in cilia function. When the area under the curve is correlated to intracellular cAMP concentration, a

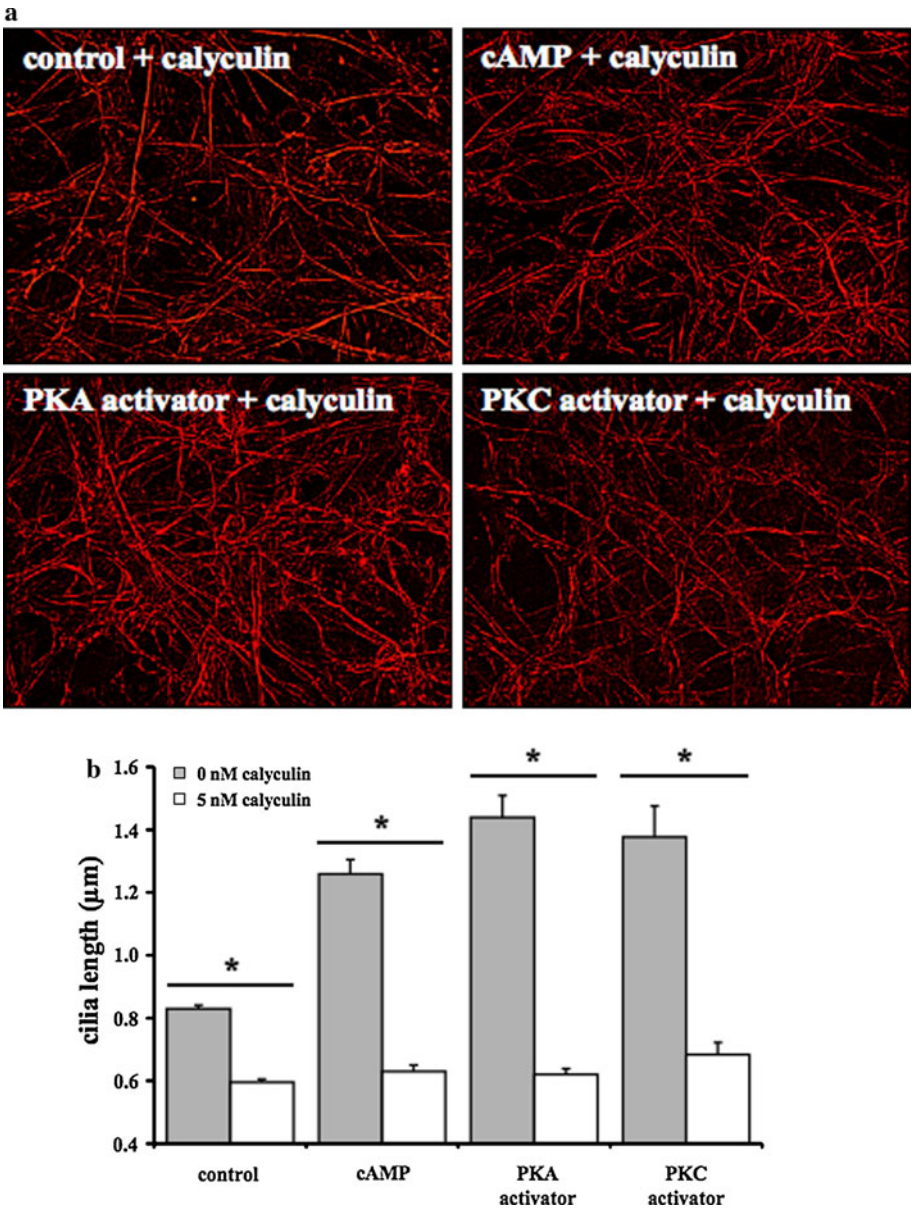

Fig. 4 Inhibition of actin rearrangement is sufficient to shorten ciliary length. **a** Cytoskeletal actin filament was analyzed in cells before and after treatment with calyculin, a protein phosphatase-1 inhibitor. Regardless of the absence or presence of cAMP analog, PKA activator, or PKC activator, filamentous actin stress fibers are always observed in cells treated with calyculin. **b** Calyculin-induced actin stress fiber maintenance is accompanied by a shortening in cilia length. $n = 3$ independent experiments. $^{*}p < 0.05$

good correlation with R^2 of 0.81 is observed (Fig. 5c). For the first time, we show that cilia length and function are not regulated in the same precise manner.

Discussion

For the first time, we show that endothelial cilia can be regulated through intracellular cAMP, cAMP-dependent protein kinase (PKA), calcium-dependent protein kinase (PKC), and mitogen-activated protein kinase (MAPK) in mono-layered cells in vitro and femoral arteries ex vivo. We further show that protein phosphatase-1 (PP-1), cofilin and actin rearrangement are also involved in regulating cilia length. Interestingly, only intracellular cAMP and PKA are involved in regulating both cilia length and function. We thus propose that cilia length and function can be regulated by distinct, yet complex intertwined signaling pathways (Fig. 6).

Primary cilium functions as a sensory organelle and houses a large number of specialized proteins. These specialized proteins are involved in various sensing of fluid-shear stress, chemical, photon, gravity, and many others, which function may be dependent or independent from extracellular calcium influx [2, 17]. Most importantly, these sensory proteins are not synthesized within the ciliary body. Intraflagellar transport (IFT) has been known to be responsible for ciliary function and growth, by carrying these sensory proteins and ciliary building blocks from the cell body to the ciliary body. Thus, the structural length and

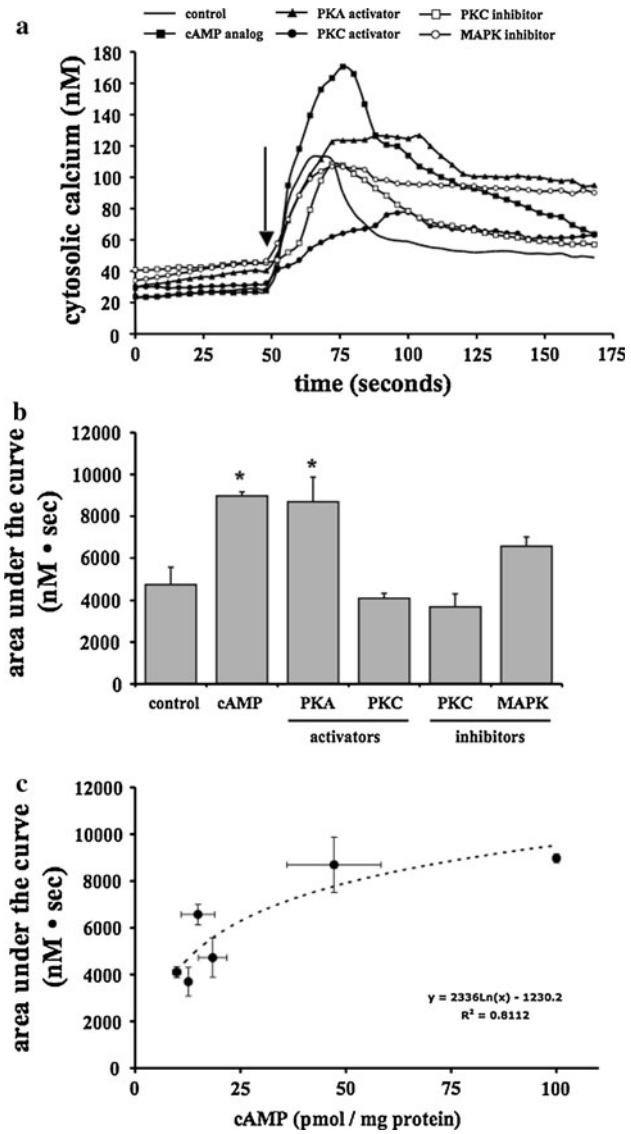

Fig. 5 Cilia function is regulated by cAMP-PKA activity. **a** Changes in cytosolic calcium in response to fluid-flow shear stress were determined in untreated cells (control) and cells treated with 8-pCPT-cAMP (cAMP analog), forskolin (PKA activator), PMA (PKC activator), bisindolylmaleimide (PKC inhibitor), or PD98059 (MAPK inhibitor). *Arrow* indicates the start of fluid shear stress. **b** Cilia function quantified through total changes in cytosolic calcium is indicated as area under the curve (nM s). Activation of cAMP-dependent protein kinase considerably promotes cilia function. **c** Intracellular cAMP concentrations (pmol/mg protein) shows a functional correlation with cilia function, indicated as area under the curve (nM s). $n \geq 3$ independent experiments; each represents an average of 100–150 cells

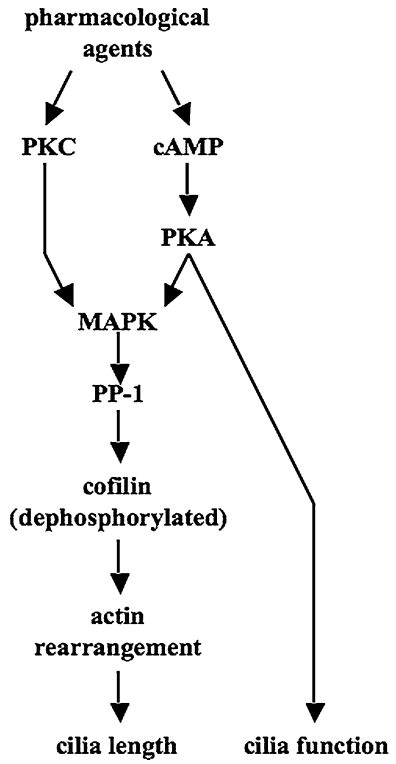

Fig. 6 Cilia length and function are hypothesized to a complex divergence cellular pathway. Our working model predicts that both calcium- and cAMP-dependent protein kinases (PKC and PKA) are involved in regulating cilia length through MAP kinase (MAPK) and protein phosphatase-1 (PP-1). Our data further indicates that PP-1 plays an important role in actin rearrangement, which is a requirement for cilia length regulation. In addition to its role in cilia length modulation, PKA is also involved in regulating cilia function. Because PKC and MAPK are not involved in regulating cilia function, we propose that cilia length and function can be regulated by complex intertwined signaling pathways

functional maintenance of primary cilium depends on proper functionality of IFT or IFT-associated molecules [18, 19].

To further determine the mechanisms involved in ciliary growth and function, we used various well-recognized pharmacological agents in mouse aortic endothelial cells that have been previously generated and characterized [6, 7]. Our data suggest that cilia length can be increased by cAMP levels and PKA activation followed by MAPK activation. Interestingly, MAPK is involved in regulating IFT. MAPK is involved in phosphorylation of motor proteins and/or linker proteins associated with IFT [20]. In addition, MAPK has been proposed to play a similar role in both flagellar biogenesis and the sensory abilities of flagella in *trypanosomatids* and *C. elegans* [21, 22]. This opens the possibility that MAPK could regulate both the length of the primary cilium and its ability to function as a sensory organelle. Thus, inhibition of MAPK is expected to result in inefficient IFT mechanism resulting in decreased cilia length, as observed in our experiments.

Our study further shows that PKC activation can also induce MAPK activity, thereby increasing cilia length. In addition, MAPK can induce PP-1 activity [23], which in turn activates cofilin [14]. Activation of cofilin is accompanied by filamentous actin rearrangement. The

involvement of PP-1 and its substrate cofilin on actin rearrangement is further reinforced by the fact that inhibiting activity of PP-1 not only reverses the effects of MAPK but also sufficiently decreases the cilia length at the basal level. Consistent with this finding, modifying the stability of actin cytoskeleton has been implicated in cilia growth [24, 25].

Worth discussing is that activation of PKC tends to decrease cAMP, yet PKC activation increases cilia length. We propose that PKC-induced cilia length increase does not require cAMP/PKA-signaling pathway. However, it has been reported that PKC can also interact and activate various phosphodiesterases (PDEs). PDEs are known to hydrolyze cAMP, resulting in a low level of intracellular cAMP [26]. We thus believe that PKC/PDE/cAMP is a secondary pathway, while PKC/MAPK/PP-1 is the primary signaling pathway for cilia length regulation. Supporting our idea, PKC has been shown to directly activate MAPK activity [27], and MAPK is known to activate PP-1 [23].

PP-1 is a versatile phospho-Ser/Thr phosphatase involved in various eukaryotic cellular functions. Furthermore, PP-1 has been implicated in actin rearrangement in non-muscle cells [28]. At least in the endothelial cells, our data suggest the involvement of cofilin as the actin-related or actin-binding protein (ARP/ABP) involved in actin rearrangement. Of note is that cofilin is one of many PP-1 substrates [14]. Once activated by PP-1, cofilin is readily recruited to the leading edge of the actin filament, resulting in re-organization of the filament [29]. Whether other ARPs/ABPs would be involved in regulating cilia length remains to be a possibility in other systems, such as in renal epithelial cells.

In addition to IFT, inhibition of actin polymerization is thought to have a major role in ciliogenesis. Actin dynamics, for example, plays an important role in ciliogenesis [24, 25]. In addition, a thick plank of actin at the apical surface of cells is required for docking of the basal body and the subsequent formation of the ciliary axoneme [30]. Thus, any activation or inhibition of actin polymerization could affect the microtubule-based cilium [31], which is in agreement with our current study.

It has been reported that activation of the Raf/MEK/ERK pathway is accompanied by actin cytoskeletal reorganization, and cells with ciliary dysfunction exhibit altered actin-spindle organization resulting in substantially centrosomal overamplification and polyploidy [32]. Cofilin is an essential regulator of actin dynamics, participating in reorganization actin cytoskeletal structure among other cellular processes. Cofilin activity is also regulated by Ras/MEK/ERK pathway via PP-1. Ras/MEK/ERK can phosphorylate and activate PP-1, which in turn dephosphorylates and activates cofilin [14, 23]. Thus, treatment of our endothelial cells with MAPK inhibitor, PD98059, and PP-1 inhibitor, calyculin, would result in similar consequences, i.e., loss of actin rearrangement and subsequent decrease cilia length.

The primary cilium is structurally composed of acetylated and detyrosinated microtubules. Thus, activation or inhibition of any cell cytoskeletal proteins can affect the function of microtubule-based cilium [31]. Though our data suggest that MAPK/PP-1/cofilin may play an important role in cilia length, cilia function does not seem to be regulated by this mechanism. PKC activation, which promotes an increase in cilia length, also does not seem to play any role in cilia function. Our functional studies indicate that activation of cAMP/PKA pathway can promote an increase in cilia function, in addition to cilia length. Overall, our study concludes that the molecular interactions between cilia function and length can be independent of one another. Furthermore, increasing cilia length does not necessarily translate into an increase in cilia function, and vice versa.

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