Epac as a novel regulator of airway smooth muscle phenotype and function

Roscioni, Sara Silvia

Compartmentalization of cAMP signaling in human airway smooth muscle cells

Sara S. Roscioni, Clelia Iovino, Mark H. Menzen, Reinoud Gosens, Andrew J. Halayko, Herman Meurs, H. Maarsingh, Martina Schmidt

Chapter 6

Abstract

Accumulation of cAMP is driven by G_s protein-coupled receptors and reaches a distinct subset of cellular substrates partly embedded in A-kinase anchoring proteins (AKAPs)-dependent complexes and/or caveolae-enriched domains. In such multidomain structures, the cAMP effectors protein kinase A (PKA) and exchange proteins directly activated by cAMP (Epac1 and Epac2) may participate in cAMP-mediated responses, including cytokine release. In airway smooth muscle (ASM) cells, Epac and PKA potentiate the release of interleukin-8 (IL-8) induced by the inflammatory mediator bradykinin in a Rap1-dependent manner (**chapter 4**). However, compartmentalization of Epac- and PKA-mediated effects in ASM cells has not been studied yet. Using immortalized human ASM cells, we here investigated subcellular localization and potential clustering of cAMP effectors with AKAPs and caveolae, lipid membrane invaginations implicated in ASM functioning. In resting cells, Epac1 and Epac2 are differentially distributed through the cell. Epac1, but not Epac2, translocated to the membrane and the nucleus upon specific activation. Interestingly, Epac1, Epac2, PKA, AKAP79 and Rap1 localized in caveolae-enriched membrane fractions. By using a cholesterol-depleting agent that disrupts caveolae, we show that caveolae are crucial for bradykinin-induced IL-8 release. In addition, the PKA-AKAP-binding disrupting peptide Ht31 blocked the PKA- and Epac-dependent augmentation of bradykinin-induced IL-8 release, indicating that AKAP-multiprotein complexes may control cAMP-mediated regulation of ASM inflammatory functions.

Introduction

Cyclic adenosine monophosphate (cAMP) is a highly diffusing molecule which is generated from adenosine triphosphate by the action of adenylyl cyclases (ACs) [1] upon stimulation of G_s-protein coupled receptors (G_sPCR). Intracellular levels of cAMP are tuned by the action of phosphodiesterases (PDEs), which hydrolyze cAMP and shape its gradients throughout the cell [2]. Elevations of cAMP subsequently activate the downstream effectors protein kinase A (PKA) and exchange proteins directly activated by cAMP (Epac). Activation of Epac and/or PKA generates different functional effects and even in identical cellular settings activation of the two cAMP effectors may act multifunctionally [3-5]. Epac and PKA are activated at similar cAMP concentrations [6], interact with similar effector proteins [5] (**chapter 4**), and may act alone [7] or either synergize [8] or antagonize each other [9]. Hence, questions have raised about the specificity of cAMP to integrate diverse extracellular signals by eliciting appropriate functional responses.
An emerging explanation is represented by signal compartmentalization. In particular, new evidence suggests that cAMP signaling specificity is achieved through assembly of distinct multiprotein complexes at specific cellular locations by anchoring and scaffolding proteins [10]. For example, A-kinase anchoring proteins

(AKAPs) bind to PKA via their PKA-binding domain and target PKA to defined subcellular compartments via a unique domain [11]. Different AKAPs mediate specific cellular functions due to their capacity to form multiprotein complexes by interaction with kinases/phosphatases, ACs, PDEs, and other cAMP signaling components [12, 13]. Hence, AKAPs provide a high level of spatio-temporal control of cAMP signaling by coordinating the actions of distinct enzymes and targeting them to compartmentalized pools of cAMP. Recently, Epac has been shown to interact with AKAP complexes providing a dual control of cAMP functions [14, 15]. Moreover, specific subcellular localization of Epac and its downstream targets seems to correlate with their distinct cellular functions [16]. An important cellular compartment is represented by caveolae, omega-shaped cholesterol-enriched membrane invaginations believed to importantly participate in signal transduction [17-19]. Although upstream cAMP regulators such as G_sPCRs, ACs and PDEs have been shown to (co)localize with caveolae [19, 20], association of Epac with caveolae has not been studied yet.

Airway smooth muscle (ASM) cells play a key role in lung physiology as well as in the pathogenesis and treatment of airway obstructive diseases. ASM cells express numerous caveolae, which modulate several cellular functions, including proliferation, contraction and phenotypic changes, upon clustering and regulation of distinct signaling molecules [21]. Recently, we have shown that elevation of cAMP and activation of Epac and PKA potentiates the release of interleukin-8 (IL-8) from human ASM cells induced by the inflammatory mediator bradykinin via activation of the small GTPase Rap1 [5] (**chapter 4**). However, the role of compartimentalization of cAMP signaling in this process in currently unkown. Hence, studies on cAMP compartmentalization in ASM cells would provide new insights into the signaling specificity of this multipotent molecule and may help to improve the therapeutical benefit of airway disease medications. Using human ASM cells, we investigated the subcellular (re)distribution of Epac and its association to AKAPs and caveolae as well as the functional consequences of these mechanisms on bradykinin-induced IL-8 release.

Materials and methods

Materials. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin solution and amphotericin B solution (fungizone) were obtained from GIBCO-BRL Life Technologies (Paisley, UK). Trypsin EDTA was purchased from Lonza (Basel, Switzerland). 8-pCPT-2'-O-Me-cAMP and 6-Bnz-cAMP were from BIOLOG Life Science Institute (Bremen, Germany). Fenoterol hydrobromide was from Boehringer Ingeheim (Ingelheim, Germany). Methyl-β-cyclodextrin, apoprotein, leupeptin, pepstatin, Na_3VO_4, β-glycerophosphate, anti-mouse, anti-rabbit and anti-goat secondary antibodies, sucrose and Triton X-100 were from Sigma-Aldrich (St. Louis, USA). The Pierce BCA protein assay kit was from Thermo Scientific (Rockford, USA). Antibodies against Epac1 and Epac2 were kindly provided by Dr. J. L. Bos (University Medical Center Utrecht, The Netherlands). The protein-A agarose and antibodies against GAPDH, caveolin-1, Rap-1, lamin-A were from Santa Cruz Biotechnology (Santa Cruz, USA). Antibodies against AKAP79 and PKA RII were from BD Biosciences (Sparks, USA). Western lightning ECL solution was from PerkinElmer (Waltman, USA) and Prolong Gold Antifade reagent, Hoechst, FITC and CY3 from InVitrogen (Carlsbad, USA). IL-8 ELISA kit was from Sanquin (Amsterdam, The Netherlands) and Alamar Blue solution was from Biosource (Camarillo, USA). All other reagents were of analytical grade.

Cell culture. Human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT-ASM cells) were used for all experiments [17, 22]. Cells were grown in DMEM supplemented with 50 U/ml streptomycin, 50 µg/ml penicillin, 10% (v/v) FBS and 3 ml fungizone (1.5 µg/ml). Cells were grown to 100% confluency and kept for one day in serum-free DMEM supplemented with antibiotics before each experiment.

Cell fractionation. Cells grown onto 150 mm dishes were first stimulated with 8-pCPT-2'-*O*-Me-cAMP (100 µM) for 15 min and then washed twice with ice-cold PBS and lysed for 5 minutes in 50 mM Tris (pH 7.4) supplemented with 1 mM Na_3VO_4, 1 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin and 1,06 mg/ml β-glycerophosphate. After 10 strokes in a Potter homogenizer, the homogenate was collected and centrifuged for 5 min at 1000 *g*. The solid phase, containing nuclei and cytoskeletal fractions, was then further purified by treatment with 250 µl 1% v/v Triton X-100 in order to solubilize the nuclei and remove the insoluble cytoskeletal fraction by centrifugation for 5 min at 210000 *g*. The supernatant obtained in the initial centrifugation step, containing both the membrane and the cytosolic fractions, was centrifuged for 30 min at 210000 *g*. The cytosol-enriched supernatant was collected and the membrane-enriched pellet was resuspended in 170 µl of RIPA buffer (composition: 40 mM Tris, 150 mM NaCl, 1% v/v Igepal CA-630, 1% wt/vol deoxycholic acid, 1 mM NaF, 1 mM Na_3VO_4, 10

μg/ml aprotinin, 10 μg/ml leupeptin and 7 μg/ml pepstatin A, pH 8.0), sonicated and stored at -20°C until further use. The protein amount of all the fractions was determined using Pierce protein determination, according to the manifacturer's instructions. Membrane, cytosolic and nuclear enriched fractions were subsequently used for detection of Epac1 and Epac2 expression.

Isolation of caveolae membrane fractions. Cells grown onto 150 mm dishes were stimulated with 8-pCPT-2'-*O*-Me-cAMP (100 μM for 15 min and then washed with ice-cold PBS and lysed in 500 mM sodium carbonate (pH 11.0) supplemented with 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, and 1,06 mg/ml β-glycerophosphate. Caveolae were isolated as previously described [23]. After scraping, 2 ml of homogenate was mixed with an equal volume of 80% (w/v) sucrose and placed in the bottom of a centrifuge tube. A stepwise sucrose density gradient (40%, 30%, 20%, and 5%) was then carefully layered on top of the homogenate. Thereafter, the samples were centrifuged at 210000 *g* for 18 hrs at 4°C, and then 12 fractions of 1 ml each were collected from the top of the gradient. Samples were stored at -20°C.

Western blot analysis. Equal amounts of protein were loaded on 8-15% polyacrylamide gels, transferred to nitrocellulose membranes and analyzed for the protein of interest by using the specific first antibody (dilution Epac1 1:500, Epac2 1:500, Caveolae 1:1000, Rap1 1:100, AKAP79 1:250, PKA 1:1000) and the secondary HRP-conjugated antibody (dilution anti-rabbit 1:2000, anti-mouse1:3000, anti-goat 1:10000). Protein bands were subsequently visualized using western lightning plus-ECL on film or by using the G- box (SYNGENE, Frederick, USA) and quantified by scanning densitometry using TotalLab software (Nonlinear Dynamics, Newcastle, UK). Results were normalized for protein levels by using specific control proteins.

Immunofluorescence. Cells were seeded in coverslips (10000/well) and stimulated with 8-pCPT-2'-*O*-Me-cAMP (100 μM for 15 min. Cells were fixed for 15 min with a solution containing 3% paraformaldehyde, permealized with a solution of 3% paraformaldehyde plus 0,3% Triton X-100 for 5 min, and then immunolabeled using anti-caveolin-1, anti-Epac1, anti-Epac2, anti-Rap1 and anti-AKAP79. Antibodies were diluted 10 times compared to the dilutions used for Western Blotting. For negative controls, coverslips were incubated in the same buffer containing no primary antibody. Donkey anti-rabbit FITC or donkey anti-mouse Cy3 conjugated secondary antibodies were used to detect primary antibody bound to fixed cells. Nuclei were stained with Hoechst 33342 (0,1 μg/ml, dilution 1:10000). Coverslips were mounted using 10 μl/well ProLong Gold antifade reagent and analyzed using an Olympus AX70 microscope equipped with digital image capture system

(ColorView Soft System with Olympus U CMAD2 lens). 400 times magnification was used.

Protein complex immunoprecipitation. Cells were cultured in 100 mm Petri dishes and serum deprived at 100% confluence for 24 hrs. Then, cells were stimulated with 8-pCPT-2'-*O*-Me-cAMP (100 µM) for 15 min, lysed with 1 ml Ripa buffer and stored at -20°C. Anti-caveolin-1 (dilution 1:100) was conjugated to protein-A agarose beads by incubating overnight at 4°C with gentle rotation. The day after, 200 µl cell lysate or lysis buffer (negative control) was added to 30 µl beads with antibody and mixed overnight at 4°C with gentle rotation. The immunoprecipitates were washed with PBS by centrifugation (twice at 2,000 *g*; 5 min; at 4ºC). 50 µl 4 x loading buffer was added to each immunoprecipitate and samples were boiled for 5 min. 40 µl samples were loaded for the detection of Rap1 and caveolin-1.

ELISA. Cells were seeded in 24 well plates and treated with 8-pCPT-2'-*O*-Me-cAMP (100 µM) or 6-Bnz-cAMP (500 µM) for 10 minutes before the addition of bradykinin (10 µM). Supernatant were collected after 18 hrs. The concentration of IL-8 in the culture medium was determined by ELISA according to the manifacturer's instructions (Sanquin, the Netherlands). In some experiments, cells were pre-treated for 1 hr with the cholesterol-depleting agent methyl-β-cyclodextrin (5 mM), followed by two washes in DMEM before stimulation. Alternatively, the PKA-AKAP-binding disruptor peptide Ht31 and the negative control Ht31P (50µM, each) were added 30 min before stimulation.

Alamar Blue assay. Plates were washed twice with PBS and then 250 µl HBSS [composition (g/L): $CaCl_2$ 0.14, NaCl 8.0, KCl 0.4, KH_2PO_4 0.06, $MgCl_2.6H_2O$ 0.10, Na_2HPO_4 0.048, $MgSO_4.7H_2O$ 0.10, $NaHCO_3$ 0.35; pH 7.4] containing 5% (vol/vol) Alamar blue solution were added to each well. Plates were incubated for about 45 min at 37°C and analyzed with Wallac 1420 Victor 2TM at 590 nm.

Statistical analysis. Data were expressed as the mean ± SEM of *n* determinations. Data were compared by using an unpaired or paired two-tailed Student's t test as appropriate to determine statistical significant differences. *P* values<0.05 were considered to be statistically significant.

Results

Epac1 translocates to membranes and nucleus.

By using western blot (Fig 1A) and immunocytochemistry techniques, we found that Epac1 is ubiquitously distributed in hTERT-ASM cells (Figs. 1-3, see appendix), whereas Epac2 expression appeared higher in the cytosol and the membrane and was hardly visible in the nucleus (Figs. 1 and 4, see appendix).

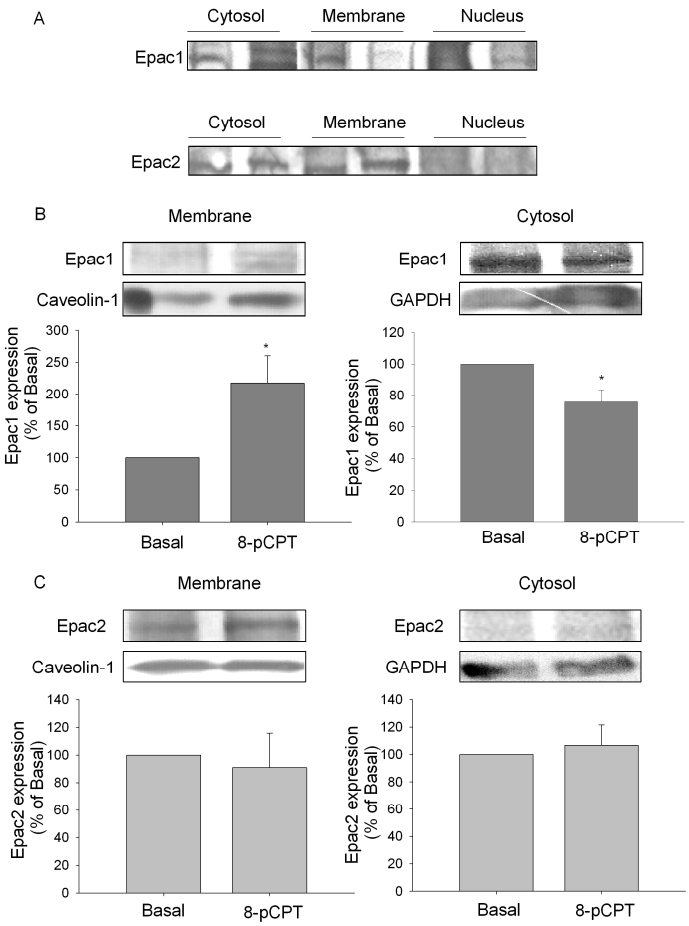

Fig. 1. (A) Epac1 and Epac2 expression in cytosol, membranes and nucleus of hTERT-ASM cells. (B) Epac1 and (C) Epac2 expression in membranes and cytosol of hTERT-ASM treated without (Basal) or with 8-pCPT-2'-O-Me-cAMP (8-pCPT, 100 μM, 10 min). Data represent means ± SEM of 3-5 independent experiments. Representative immunoblots with quantifications are shown. Epac1 and Epac2 were normalized to the appropriate control protein (caveolin-1 for membrane and GAPDH for cytosol). $^{*}P<0.05$ compared to Basal.

Immunostaining of hTERT-ASM cells revealed that after stimulation with the Epac activator 8-pCPT-2'-*O*-Me-cAMP, Epac1 translocated from the cytosol to the plasma membrane (Fig. 2B, see appendix). Accordingly, the abundance of Epac1 increased at membrane fractions ($P<0.05$, Fig 1B, left) and decreased in cytosolic fractions (Fig 1B, right), whereas no changes were observed for Epac2 after stimulation with 8-pCPT-2'-*O*-Me-cAMP (Fig 1C). Importantly, the Epac activator

also significantly increased Epac1 accumulation in the nuclear fraction (P<0.05, Fig 2), as revealed by western blot (Fig. 2A) and immunostaining (Fig. 2B, see appendix).

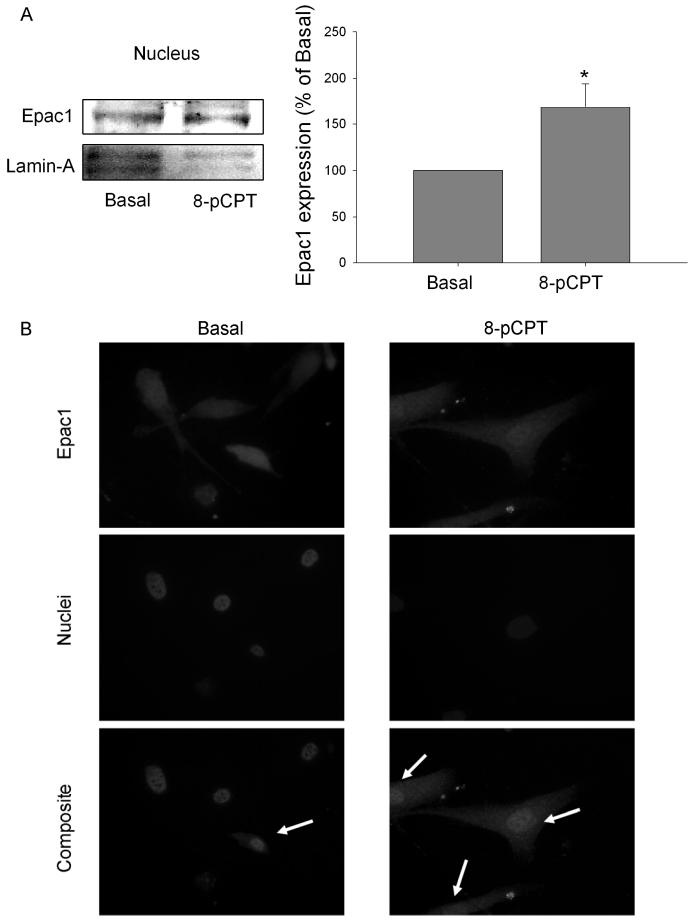

Fig. 2. (A) Epac1 expression in nuclear fractions of hTERT-ASM cells treated without (Basal) or with 8-pCPT-2'-O-Me-cAMP (8-pCPT, 100 µM, 10 min). Data represent means ± SEM of 3-5 independent experiments. Representative immunoblots with quantifications are shown. Proteins were normalized to the nuclear marker lamin A. $^{*}P$<0.05 compared to Basal. (B) Immunocytochemistry showing staining for nuclei (blue) and Epac1 (red) in hTERT-ASM cells treated without (Basal) or with 8-pCPT (100 µM, 10 min). Composite image is depicted underneath. Arrows indicate the nuclear localization of Epac1.

This was confirmed by the violet colour observed in the nuclei after merging of the blue nuclear die Hoechst with the red Cy3-labeled secondary antibody for Epac1 (Fig. 2B).

cAMP effectors and scaffolding proteins localize to caveolae microdomains. As illustrated in Fig. 3A using sucrose density gradient centrifugation, Epac1 was partially associated to caveolae in hTERT-ASM cells. These results were confirmed by immunocytochemistry by using caveolin-1 and Epac1 antibodies (Fig. 3B, see appendix).

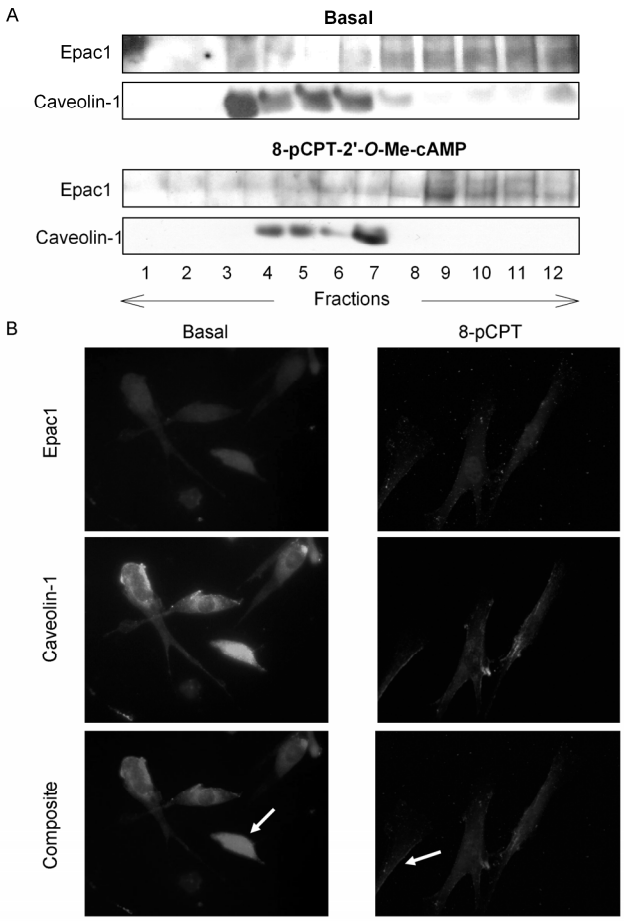

Fig. 3. (A) Epac1 and caveolin-1 expression in hTERT-ASM cells treated without (Basal) or with 8-pCPT-2'-O-Me-cAMP (8-pCPT, 100 µM, 10 min) after caveolae isolation following sucrose density centrifugation. Representative immunoblots of 5-6 independent experiments are shown. (B) Immunocytochemistry showing staining for Epac1 (red) and caveolin-1 (green) in hTERT-ASM cells treated without (Basal) or with 8-pCPT (100 µM, 10 min). Composite image is depicted underneath. Arrows indicate co-localization of Epac1 and caveolin-1.

As expected, caveolin-1 staining was clearly marked at the edge of the cells [17], whereas basal Epac1 expression exhibited a diffuse staining (Fig. 3B). Co-staining of Epac1 and caveolin-1 further support their co-localization at caveolae domains (Fig 3B). In contrast, Epac2 appeared not to co-localize with caveolin-1, although results were clouded by the overall low detection level of Epac2 (Fig 4, see appendix). For both Epac1 and Epac2, there were no clear differences in the association to caveolae after stimulation with 8-pCPT-2'-*O*-Me-cAMP compared to basal (Figs. 3 and 4).

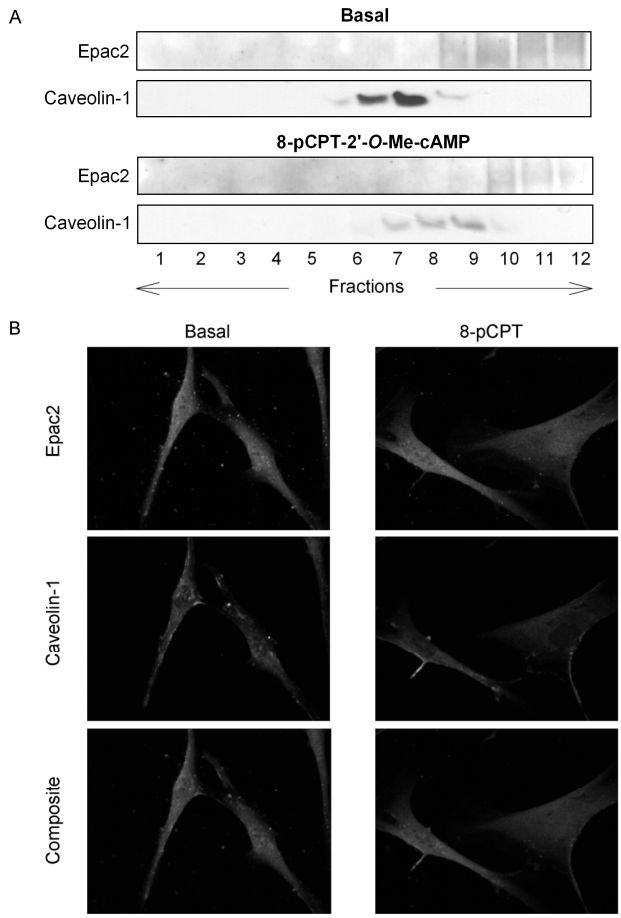

Fig. 4. (A) Epac2 and caveolin-1 expression in hTERT-ASM cells treated without (Basal) or with 8-pCPT-2'-O-Me-cAMP (8-pCPT, 100 µM, 10 min) after caveolae isolation following sucrose density centrifugation. (B) Immunocytochemistry showing staining for Epac2 (red) and caveolin-1 (green) in hTERT-ASM cells treated without (Basal) or with 8-pCPT (100 µM, 10 min). Composite image is depicted underneath. Arrows indicate co-localization of Epac2 and caveolin-1.

In resting hTERT-ASM cells, PKA, AKAP79 and particularly the Epac effector Rap1 were found to be partially expressed in caveolae fractions (Fig 5A). The association of Rap1 to caveolae was confirmed by using immunoprecipitation with caveolin-1 antibody (Fig 5B). This association, however, appeared rather insensitive to Epac activation (Fig 5B).

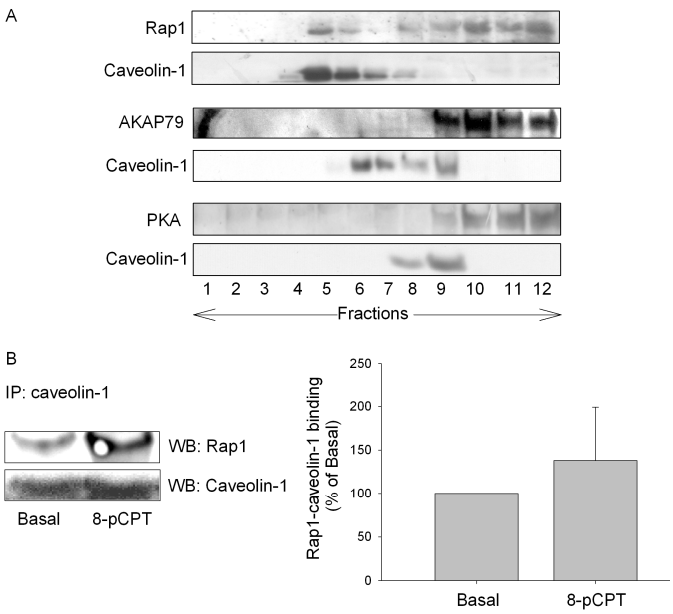

Fig. 5. (A) Expression of Rap1, PKA, AKAP79 and caveolin-1 in hTERT-ASM cells treated without (Basal) or with 8-pCPT-2'-O-Me-cAMP (8-pCPT, 100 µM, 10 min) after caveolae isolation following sucrose density centrifugation. (B) Cell lysates of hTERT-ASM cells treated without (Basal) or with 8-pCPT (100 µM, 10 min) were immunoprecipitated (IP) for caveolin-1 and precipitates were analyzed by western blotting (WB) for Rap1 and caveolin-1. Representative immunoblots of 3 experiments are shown.

Modulation of IL-8 release by caveolae and AKAPs.

We reported previously that Epac and PKA potentiate bradykinin-induced IL-8 release from hTERT-ASM cells [5] (**chapter 4**). Similar to Epac1, PKA and Rap1, bradykinin receptors localize to caveolae [24]. Therefore, we investigated the contribution of caveolae in the bradykinin-induced IL-8 release and its modulation by Epac. In line with the above mentioned study, bradykinin increased the IL-8 release from hTERT-ASM cells, which was further enhanced after co-treatment with the Epac activator 8-pCPT-2'-*O*-Me-cAMP (Fig 6A). To destroy the caveolar compartments, hTERT-ASM cells were treated with the cholesterol-depleting agent methyl-β-cyclodextrin [22]. This compound did not alter basal IL-8 production (Fig 6A) nor cell number (data not shown), suggesting that integrity of the overall cellular and membrane structure is maintained. Importantly, methyl-β-cyclodextrin completely prevented the release of IL-8 induced by bradykinin alone ($P<0.01$, Fig 6A), which could not be rescued by the Epac activator 8-pCPT-2'-*O*-Me-cAMP (Fig 6A).

Furthermore, we addressed the role of AKAPs in the cAMP-mediated regulation of bradykinin-induced IL-8 release by using Ht31, a peptide which disrupts the interaction between PKA and AKAPs with nanomolar affinity [25]. Ht31P was used as an internal control, and indeed it did not alter cellular function as revealed by the observation that bradykinin increased IL-8 release (P<0.001, Fig. 6B), a response further potentiated by 8-pCPT-2'-*O*-Me-cAMP and 6-Bnz-cAMP (P<0.01 each, Fig. 6B). Importantly, treatment with the AKAP-PKA disrupting peptide Ht31 completely prevented the Epac- and PKA-mediated augmentation of bradykinin-induced IL-8 release (P<0.05 each, Fig 6B), without affecting the effect of bradykinin alone.

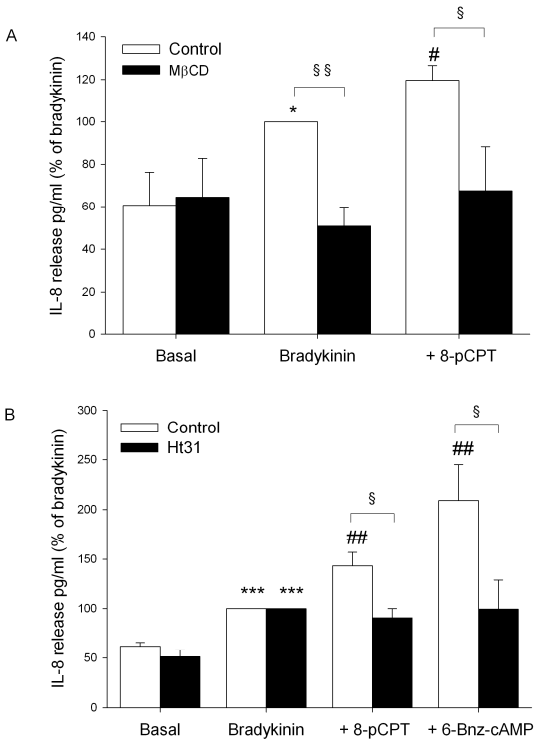

Fig. 6. (A) hTERT-ASM cells were treated for 18 hrs without (Basal) or with bradykinin (10 μM) alone or in combination with 8-pCPT-2'-O-Me-cAMP (8-pCPT, 100 μM) in the absence (white bars) or presence (black bars) of 1 hr pre-treatment with methyl-β-cyclodextrin (MβCD, 5 mM). (B) hTERT-ASM cells were treated for 18 hrs without (Basal) or with bradykinin (10 μM) alone or in combination with 8-pCPT (100 μM) or 6-Bnz-cAMP (500 μM) in presence of the PKA-AKAP disruptor Ht31 (black bars) or the control peptide Ht31P (white bars) which were added 30 min before. Data represent means ± SEM of 3-4 experiments. $^{*}P$<0.05, $^{***}P$<0.001 compared to Basal; $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared to respective control; $^{\S}P$<0.05, $^{\S\S}P$<0.01.

Discussion

Our studies represent the first description of compartmentalization of cAMP signaling in human ASM. We demonstrated that PKA, AKAP79, Epac and their main effector Rap1 associate to caveolae. Upon specific activation, Epac1, but not Epac2, translocated to the membrane and nucleus. Caveolae play a crucial role in the pro-inflammatory effect of bradykinin, whereas association to AKAP79 is necessary for proper PKA- and Epac- mediated augmentation of the bradykinin response.

The discovery of Epac as a novel cAMP effector next to PKA opened new surges in the cAMP research field and raised questions on how signalling specificity can be achieved. Recently, it was found that affinity of cAMP for Epac and the PKA holoenzyme is very similar (~2.9 μM) [6], suggesting that physiological cAMP elevation potentially activates both effectors. Epac and PKA seem to modulate similar cellular responses, but have also been shown to antagonize each other effects or to act independently [5, 7-9]. These findings indicate the occurrence of specific events. Although cell-type specific expression of PKA and/or Epac may provide an explanation, it does not justify the redundant properties and interconnectivity of Epac and PKA in the same cell type [5, 14]. The emerging view is that specificity is guaranteed by spatio-temporal organization of signaling events. Current techniques allow the visualization of cAMP microdomains within cells [26], most likely generated by the coordinated action of distinct pools of ACs and PDEs to maintain cAMP accumulations to specific cellular locations. Subcellular targeting of cAMP-related effectors has been proposed as a potential mechanisms to gain biological specificity [9, 16, 27]. Epac1 has been associated with several cellular locations - including the plasma membrane, the nuclear envelope and the cytosol - and compartmentalization is believed to reflect its multifunctionality [9, 16, 28]. Activation of Epac1 has been shown to induce its translocation to the plasma membrane, which is a prerequisite for Rap1 activation and enhancement of cell adhesion in HEK293 cells [28]. Similarly, plasma membrane localization has been reported to be essential for Epac2 signaling, where it is directly bound to activated Ras [29, 30]. Using human ASM cells, we found Epac1 to be ubiquitously distributed in resting cells. Interestingly, treatment with the Epac activator 8-pCPT-2'-*O*-Me-cAMP induced the migration of Epac1 from the cytosol to the membrane. In contrast, Epac2 was found to be constitutively present at membrane and cytosolic fractions and insensitive to activation by 8-pCPT-2'-*O*-Me-cAMP. Hence, different mechanisms of cellular targeting may distinguish the roles of Epac1 and Epac2 and give insights into their specific biological functions. Nuclear localization of Epac1 has been previously described [9, 16] and implicated in the regulation of cell division by Epac1 [16]. Indeed, Huston and co-workers recently reported that nuclear localization of Epac1 regulates nuclear/cytosolic trafficking of DNA-dependent protein kinase [9]. We report here that the Epac activator 8-pCPT-2'-*O*-Me-cAMP induces translocation of Epac1 to the nucleus in ASM cells, a process that might control cellular function. In support, we showed that next to bradykinin-induced IL-8 release, Epac regulates IL-8 release induced by cigarette smoke extract (**chapter 5**) and inhibits growth-factor induced ASM proliferation upon activation of extracellular signal-regulated kinases 1/2 [31] (**chapters 8 and 9**). Taken together, these findings suggest that (stimulus) specific translocation of Epac via different anchors/domains allows Epac to reach compartmentalized pools of substrates and to provide the specific biological outcome.

We report here for the first time that Epac and Rap1 localize to caveolae in ASM cells. In particular, adrenoreceptors (β_1- and β_2) and a subset of ACs have been previously found to (co)localize with caveolae and such interactions seem to affect receptor-coupling efficiency to ACs [19]. Caveolae regulate several ASM cellular functions including cell proliferation, inflammation and phenotypic changes [21], upon clustering and subsequent regulation of signaling molecules, including bradykinin receptors [24]. Since Epac/Rap1 also associate to caveolae, this complex may play an important role in the cAMP-mediated regulation of ASM functions. In order to characterize Epac and Rap1 functional association to caveolae, we measured Epac-mediated effect on bradykinin-induced IL-8 release in the presence of a caveaole-disrupting agent. This treatment completely blunted the bradykinin-induced IL-8 release, demonstrating that the association to caveolae is crucial for bradykinin receptors to regulate cytokine release in ASM. The same pharmacological approach previously implicated caveolae in muscarinic receptor-mediated intracellular Ca^{2+} mobilization and ASM contraction [18]. However, bradykinin-mediated effects on Ca^{2+} modulation resulted insensitive to caveolae disruption [18]. Hence, these data point at a functional-specific role for caveolae in ASM, and suggest that caveolae localization may differentially affect receptor-mediated cellular responses in ASM. Co-activation of Epac did not rescue the effect of methyl-β-cyclodextrin on bradykinin-induced IL-8 release, demonstrating that Epac requires a proper bradykinin signaling in order to increase cytokine release from ASM. This is in line with our previous findings, demonstrating that 8-pCPT-2'-*O*-Me-cAMP by itself does not affect IL-8 release from ASM [5] (**chapter 4**).

Another mean of cAMP compartmentalization is represented by AKAPs, which indeed represent PKA-anchoring proteins [32]. AKAPs guarantee PKA signaling specificity upon clustering of a specific subset of substrates and targeting them to distinct cellular compartments [32, 33]. Several studies described Epac to be part of AKAP complexes, providing dual control of cAMP-mediated functions [14, 15]. Thus, a muscle specific AKAP coordinates the action of PKA and Epac1 in the regulation of cardiac hypertrophy in cardiomyocytes [14], whereas PKA and Epac2 associate to AKAP79 to differentially regulate protein kinase B/Akt phosphorylation in neuronal cells [15]. We found that next to Epac and Rap1, PKA and AKAP79 also localize to caveolae in ASM cells, suggesting that these proteins may form a complex within the caveolae. Importantly, after disruption of PKA-AKAP79 binding, the bradykinin-induced IL-8 release was no longer augmented by activation of PKA or of Epac, even though the effect of bradykinin by itself was unaffected. These findings support that PKA and Epac are interconnected with respect to their regulation of cytokine release from ASM as previously described using pharmacological and RNA silencing approaches [5] (**chapter 4**). In addition, our findings indicate the existence of an AKAP-dependent complex, which tethers Epac and PKA and coordinates their interconnectivity in regulating bradykinin-induced IL-8 release. In conclusion, our findings demonstrate the existence of

compartmentalization of cAMP signaling in ASM, which functionally regulates Epac- and PKA-mediated responses in these cells. Caveolae play a crucial role in the pro-inflammatory actions of bradykinin. Subcellular localization of cAMP effectors to caveolae may play an important role in regulation of airway function as well, and nuclear translocation of activated Epac may serve as a mean to control DNA-dependent events. Moreover, AKAP appears a novel regulator of Epac and PKA-mediated inflammatory effects in ASM.

References

1. Hunter T: **Signaling--2000 and beyond.** *Cell* 2000, **100:** 113-127.
2. Conti M, Beavo J: **Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling.** *Annu Rev Biochem* 2007, **76:** 481-511.
3. Cheng X, Ji Z, Tsalkova T, Mei F: **Epac and PKA: a tale of two intracellular cAMP receptors.** *Acta Biochim Biophys Sin (Shanghai)* 2008, **40:** 651-662.
4. Grandoch M, Roscioni SS, Schmidt M: **The role of Epac proteins, novel cAMP mediators, in the regulation of immune, lung and neuronal function.** *Br J Pharmacol* 2010, **159:** 265-284.
5. Roscioni SS, Kistemaker LE, Menzen MH, Elzinga CR, Gosens R, Halayko AJ, Meurs H, Schmidt M: **PKA and Epac cooperate to augment bradykinin-induced interleukin-8 release from human airway smooth muscle cells.** *Respir Res* 2009, **10:** 88-104.
6. Dao KK, Teigen K, Kopperud R, Hodneland E, Schwede F, Christensen AE, Martinez A, Doskeland SO: **Epac1 and cAMP-dependent protein kinase holoenzyme have similar cAMP affinity, but their cAMP domains have distinct structural features and cyclic nucleotide recognition.** *J Biol Chem* 2006, **281:** 21500-21511.
7. Enserink JM, Christensen AE, de RJ, van TM, Schwede F, Genieser HG, Doskeland SO, Blank JL, Bos JL: **A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK.** *Nat Cell Biol* 2002, **4:** 901-906.
8. Hochbaum D, Hong K, Barila G, Ribeiro-Neto F, Altschuler DL: **Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis.** *J Biol Chem* 2008, **283:** 4464-4468.
9. Huston E, Lynch MJ, Mohamed A, Collins DM, Hill EV, MacLeod R, Krause E, Baillie GS, Houslay MD: **EPAC and PKA allow cAMP dual control over DNA-PK nuclear translocation.** *Proc Natl Acad Sci U S A* 2008, **105:** 12791-12796.
10. Scott JD, Pawson T: **Cell signaling in space and time: where proteins come together and when they're apart.** *Science* 2009, **326:** 1220-1224.
11. Colledge M, Scott JD: **AKAPs: from structure to function.** *Trends Cell Biol* 1999, **9:** 216-221.
12. Coghlan VM, Perrino BA, Howard M, Langeberg LK, Hicks JB, Gallatin WM, Scott JD: **Association of protein kinase A and protein phosphatase 2B with a common anchoring protein.** *Science* 1995, **267:** 108-111.
13. Tasken KA, Collas P, Kemmner WA, Witczak O, Conti M, Tasken K: **Phosphodiesterase 4D and protein kinase a type II constitute a signaling unit in the centrosomal area.** *J Biol Chem* 2001, **276:** 21999-22002.
14. Dodge-Kafka KL, Soughayer J, Pare GC, Carlisle Michel JJ, Langeberg LK, Kapiloff MS, Scott JD: **The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways.** *Nature* 2005, **437:** 574-578.
15. Nijholt IM, Dolga AM, Ostroveanu A, Luiten PG, Schmidt M, Eisel UL: **Neuronal AKAP150 coordinates PKA and Epac-mediated PKB/Akt phosphorylation.** *Cell Signal* 2008, **20:** 1715-1724.

16. Qiao J, Mei FC, Popov VL, Vergara LA, Cheng X: **Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP.** *J Biol Chem* 2002, **277:** 26581-26586.
17. Gosens R, Dueck G, Gerthoffer WT, Unruh H, Zaagsma J, Meurs H, Halayko AJ: **p42/p44 MAP kinase activation is localized to caveolae-free membrane domains in airway smooth muscle.** *Am J Physiol Lung Cell Mol Physiol* 2007, **292:** L1163-L1172.
18. Gosens R, Stelmack GL, Dueck G, Mutawe MM, Hinton M, McNeill KD, Paulson A, Dakshinamurti S, Gerthoffer WT, Thliveris JA, Unruh H, Zaagsma J, Halayko AJ: **Caveolae facilitate muscarinic receptor-mediated intracellular Ca2+ mobilization and contraction in airway smooth muscle.** *Am J Physiol Lung Cell Mol Physiol* 2007, **293:** L1406-L1418.
19. Ostrom RS, Gregorian C, Drenan RM, Xiang Y, Regan JW, Insel PA: **Receptor number and caveolar co-localization determine receptor coupling efficiency to adenylyl cyclase.** *J Biol Chem* 2001, **276:** 42063-42069.
20. Schwencke C, Okumura S, Yamamoto M, Geng YJ, Ishikawa Y: **Colocalization of beta-adrenergic receptors and caveolin within the plasma membrane.** *J Cell Biochem* 1999, **75:** 64-72.
21. Halayko AJ, Tran T, Gosens R: **Phenotype and functional plasticity of airway smooth muscle: role of caveolae and caveolins.** *Proc Am Thorac Soc* 2008, **5:** 80-88.
22. Gosens R, Stelmack GL, Dueck G, McNeill KD, Yamasaki A, Gerthoffer WT, Unruh H, Gounni AS, Zaagsma J, Halayko AJ: **Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle.** *Am J Physiol Lung Cell Mol Physiol* 2006, **291:** L523-L534.
23. Smart EJ, Ying YS, Mineo C, Anderson RG: **A detergent-free method for purifying caveolae membrane from tissue culture cells.** *Proc Natl Acad Sci U S A* 1995, **92:** 10104-10108.
24. Prakash YS, Thompson MA, Vaa B, Matabdin I, Peterson TE, He T, Pabelick CM: **Caveolins and intracellular calcium regulation in human airway smooth muscle.** *Am J Physiol Lung Cell Mol Physiol* 2007, **293:** L1118-L1126.
25. Newlon MG, Roy M, Morikis D, Carr DW, Westphal R, Scott JD, Jennings PA: **A novel mechanism of PKA anchoring revealed by solution structures of anchoring complexes.** *EMBO J* 2001, **20:** 1651-1662.
26. Zaccolo M, Pozzan T: **Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes.** *Science* 2002, **295:** 1711-1715.
27. Niimura M, Miki T, Shibasaki T, Fujimoto W, Iwanaga T, Seino S: **Critical role of the N-terminal cyclic AMP-binding domain of Epac2 in its subcellular localization and function.** *J Cell Physiol* 2009, **219:** 652-658.
28. Ponsioen B, Gloerich M, Ritsma L, Rehmann H, Bos JL, Jalink K: **Direct spatial control of Epac1 by cyclic AMP.** *Mol Cell Biol* 2009, **29:** 2521-2531.
29. Li Y, Asuri S, Rebhun JF, Castro AF, Paranavitana NC, Quilliam LA: **The RAP1 guanine nucleotide exchange factor Epac2 couples cyclic AMP and Ras signals at the plasma membrane.** *J Biol Chem* 2006, **281:** 2506-2514.
30. Liu C, Takahashi M, Li Y, Song S, Dillon TJ, Shinde U, Stork PJ: **Ras is required for the cyclic AMP-dependent activation of Rap1 via Epac2.** *Mol Cell Biol* 2008, **28:** 7109-7125.
31. Roscioni SS, Dekkers BG, Prins AG, Menzen MH, Meurs H, Schmidt M, Maarsingh H: **cAMP inhibits airway smooth muscle phenotype modulation via Epac and PKA.** *Br J Pharmacol* 2010, **In press:**
32. Beene DL, Scott JD: **A-kinase anchoring proteins take shape.** *Curr Opin Cell Biol* 2007, **19:** 192-198.
33. Pidoux G, Tasken K: **Specificity and spatial dynamics of protein kinase A signaling organized by A-kinase-anchoring proteins.** *J Mol Endocrinol* 2010, **44:** 271-284.