



The stellate vascular smooth muscle cell phenotype is induced by IL-1 β via the secretion of PGE₂ and subsequent cAMP-dependent protein kinase A activation



Karl Blirando^a, Régis Blaise^a, Natalia Gorodnaya^a, Clotilde Rouxel^a, Olivier Meilhac^b, Pierre Vincent^a, Isabelle Limon^{a,*}

^a Sorbonne Universités, UPMC Univ Paris 06 CNRS UMR 8256 B2A, IBPS, F-75005, Paris, France

^b Inserm U1188 Diabète Athéromatose Thérapies Réunion Océan Indien (DÉTROIT) CYROI, 2, rue Maxime Rivière, 97490 Sainte Clotilde, La Réunion, France

ARTICLE INFO

Article history:

Received 17 July 2015

Received in revised form 11 September 2015

Accepted 14 September 2015

Available online 25 September 2015

Keywords:

Vascular smooth muscle cells

IL-1 β

PGE₂

cAMP

PKA

stellate cell morphology

ABSTRACT

Atherosclerosis development is associated with morphological changes to intimal cells, leading to a stellate cell phenotype. In this study, we aimed to determine whether and how key pro-atherogenic cytokines present in atherosclerotic plaques (IL-1 β , TNF α and IFN γ) could induce this phenotype, as these molecules are known to trigger the transdifferentiation of vascular smooth muscle cells (VSMCs). We found that, IL-1 β was the only major inflammatory mediator tested capable of inducing a stellate morphology in VSMCs. This finding was confirmed by staining for F-actin and vinculin at focal adhesions, as these two markers were disrupted only by IL-1 β . We then investigated the possible association of this IL-1 β -dependent change in morphology with an increase in intracellular cAMP concentration ([cAMP]), using the FRET-based biosensor for cAMP^{TEpac}^{vv}. Experiments in the presence of IL-1 β or medium conditioned by IL-1 β -treated VSMCs and pharmacological tools demonstrated that the long-term increase in intracellular cAMP concentration was induced by the secretion of an autocrine/paracrine mediator, prostaglandin E₂ (PGE₂), acting through the EP4 receptor. Finally, by knocking down the expression of the regulatory subunit PKAR1 α , thereby reproducing the effects of IL-1 β and PGE₂ on VSMCs, we demonstrated the contribution of PKA activity to the observed behavior of VSMCs.

© 2015 Elsevier B.V. All rights reserved.

Abbreviations: 6-BNZ, N⁶-benzoyladenosine-3', 5'-cyclic monophosphate; 8-pCPT-O-Me-cAMP, 8-(4-chlorophenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate sodium salt; ApoE, apolipoprotein E; cAMP, cyclic adenosine monophosphate; CCL3 or MIP-1 α , chemokine (C-C motif) ligand 3; CCL5 or RANTES, chemokine (C-C motif) ligand 5; CCL18 or PARC, chemokine (C-C motif) ligand 18; CFP, cyan fluorescent protein; COX-2, cyclooxygenase-2; CX3CL1 or fraktalkin, chemokine (C-X₃-C motif) ligand 1; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL10, chemokine (C-X-C motif) ligand 10; CXCL2, chemokine (C-X-C motif) ligand 2; CXCL3, chemokine (C-X-C motif) ligand 3; CXCL5, chemokine (C-X₃-C motif) ligand 5; DAPI, 4',6'-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; Epac, exchange proteins directly activated by cAMP; FRET, fluorescence resonance energy transfer; FSK, forskolin; GM-CSF, granulocyte macrophage colony-stimulating factor; IBMX, isobutyl-L-methylxanthine; IFN γ , interferon γ ; IL-1 β , interleukin-1 β ; IL6, Interleukin-6; LDL, low-density lipoproteins; PBS, phosphate-buffered saline; PGE₂, prostaglandin E₂; PKA, protein kinase A; PTGES1, prostaglandin H synthase 1; qPCR, quantitative polymerase chain reaction; Rap, Ras-related protein; RhoA, Ras homolog gene family, member A; Rp-8Br-cAMPS, 8-bromoadenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer; SM-MHC, smooth muscle-myosin heavy chain; SM22, smooth muscle alpha 22; sPLA2a, secreted phospholipase A type IIa; ^{TEpac}^{vv}, exchange protein directly activated by cAMP between turquoise (T) and venus-venus (VV); TNF α , tumor necrosis factor α ; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells; YFP, yellow fluorescent protein

* Corresponding author at: 7, quai St Bernard, Bat A 5ème étage, 75005 Paris, France.

E-mail address: isabelle.limon@upmc.fr (I. Limon).

1. Introduction

Atherosclerosis is the pathological process underlying most cardiovascular diseases, including acute coronary syndrome, peripheral artery disease and ischemic stroke. It is characterized by chronic inflammation of the arterial wall at sites of blood flow disturbance, leading to the formation of atherosclerotic plaques [1]. Atherosclerosis is promoted by hypercholesterolemia, which favors the infiltration of cholesterol-containing LDL into the intima of the vessels [2], where it may be oxidized. Parietal monocytes, which differentiate into macrophages [3] and vascular smooth muscle cells (VSMCs), which have a macrophage-like phenotype, take up and destroy oxidized LDLs via their scavenger receptors. They then become foam cells, and acquire the ability to secrete several different cytokines, including tumor necrosis factor α (TNF α) and interleukin-1 β (IL-1 β) [4]. Other inflammatory cells, including natural killer cells and lymphocytes, also participate in chronic inflammation by releasing interferon γ (IFN γ), [5]. The secretion of these compounds contributes to the expansion of atherosclerotic lesions, notably by inducing medial VSMCs to undergo transdifferentiation, modifying their phenotype.

Actin polymerization is involved in the maintenance of the differentiated/contractile phenotype of VSMCs [6–8]. Disruption, defects of actin cytoskeleton protein smooth muscle 22 alpha (SM22 alpha) are associated with pro-inflammatory and chondrogenic changes to the VSMC phenotype, aggravating vascular inflammation and pathological remodeling [9,10]. The transdifferentiated VSMCs also acquire new cellular functions, including roles in migration and proliferation, and these functions are regulated by dynamic actin microfilament rearrangements that may result in changes to cell morphology [11–13]. Atherosclerosis development is thus associated with morphological changes to the intimal VSMCs, mostly resulting in the adoption of a stellate shape due to the loss of focal adhesions and the disassembly of actin stress fibers. There are very few if any stellate cells in healthy human aortas, but the number of these cells steadily increases as fatty streaks gradually develop into advanced atherosclerotic plaques [14]. The role of VSMCs in atherogenesis may have been largely underestimated, because the markers used to identify these cells may not be appropriate [15]. Stellate VSMCs were first detected in human aortas, by electron microscopy, in 1986 [14]. This phenotype is well known in hepatic fibrosis [16], but little is known about the role of stellate VSMCs in atherosclerosis and the potential mediators triggering the change in phenotype from contractile to stellate cells.

We show here that IL-1 β was the only inflammatory mediator of those tested (IL-1 β , TNF α and IFN γ , all of which are present in plaques) able to trigger the phenotypic transition from contractile to stellate cells in VSMCs. Early studies described an increase in cAMP concentration as the intracellular event leading VSMCs to adopt a stellate phenotype [17–20]. We therefore investigated the possible association of the IL-1 β -dependent transdifferentiation of VSMCs with the increase in intracellular [cAMP], using a FRET-based biosensor for cAMP (^TEpac^{VV}, [21]). This technique can be used to monitor real-time changes in intracellular [cAMP] in living cells. These experiments showed that the increase in intracellular [cAMP] in response to IL-1 β stimulation was due to the secretion of a unique autocrine/paracrine mediator, PGE₂. By interacting with the EP4 receptor, PGE₂ stimulates cAMP production, in turn activating PKA and destabilizing the actin cytoskeleton and focal adhesions.

2. Materials and methods

2.1. Reagents

DMEM, type I collagen from calf skin, L-glutamine, penicillin, streptomycin, fatty acid-free bovine serum albumin, isobutyl-L-methylxanthine (IBMX), FITC-conjugated monoclonal anti-vinculin clone hVIN-1, and monoclonal mouse anti- β -actin clone AC-15, were obtained from Sigma Aldrich, Saint Quentin Fallavier, France. Rabbit anti-hospho PKA substrate antibody (100G7E) was obtained from Cell Signaling, Leiden, The Netherlands. We purchased 8-(4-chlorophenylthio)-2'-o-methyladenosine-3', 5'-cyclic monophosphate sodium salt (8-pCPT-O-Me-cAMP), N6-benzoyladenosine-3', 5'-cyclic monophosphates (6BNZ-cAMP) and 8-bromoadenosine-3', 5'-cyclic monophosphorothioate, and Rp- isomer (Rp-8-Br-cAMPS) from BioLog, Bremen, Germany. Forskolin (FSK) was obtained from Tocris Bioscience, Bristol, UK. Fetal calf serum (FCS), Fluorobrite™ DMEM and collagenase were purchased from Gibco BRL, Cergy Pontoise, France. Elastase, and LightCycler-DNA Master Plus SYBR Green were obtained from Roche Diagnostics, Meylan, France. Alexa Fluor® 594-conjugated phalloidin, OPTI-MEM, Fluorobrite DMEM and Lipofectamine® RNAiMAX Reagent were obtained from Invitrogen, Saint Aubin, France. Oligonucleotides were obtained from Eurofins Genomics, Ebersberg, Germany. Recombinant human IL-1 β , rat TNF α and IFN γ were obtained from Peprotech, Neuilly-sur-Seine, France. The PGE2 receptor antagonists, L-798 106 (EP3R), SC51322 (EP1R) and ONO AE3208 (EP4R) were obtained from Tocris Bioscience, Bristol, United Kingdom.

2.2. Cell culture

Rats were euthanized by the *i.p.* injection of pentobarbital (300 mg/kg) and VSMCs were isolated as previously described [22]. The university ethics committee approved the experiments on rats and all animal procedures conformed to European Directive 2010/63/EU. The VSMC response to IL-1 β decreased rapidly with successive cell passages. We therefore performed all experiments on cells that had been subcultured only one to three times. We coated the wells of

Table 1
Summary of qPCR primer sequences.

Gene family	Target genes	Forward primers (5'-3')	Reverse primers (5'-3')	Accession numbers
Eicosanoids	COX-2	ggaagctcttggctcgtg	tcttgatcgtctctcatcagta	NM_017232.3
	sPLA2 IIA	atggccttggctcaat	gcaaccgtagaagccata	NM_031598.3
	PTGES	agctagggtgtggatctc	acaatcttctgtccttctgttat	NM_021583.3
Chemokines	CXCL1	gactccagccacactccaac	cagcgctgcacagagaag	NM_030845.1
	CXCL2	ggatcgctccaaaagatactgaac	ggtagcaggagcccatgttctt	NM_053647.1
	CXCL3	ggctctcaatctgcac	ggccacaacagtcctga	NM_138522.1
	CXCL5	cactgccacagcatcgag	ttaagcaaacacagcgtagctc	NM_022214.1
	CXCL10	atgaaccaagtgtctgtgt	gtctcagcgtctgttcatgg	NM_139089.1
	CX3CL1	cgtgccacaagatgacctc	ctgtgctgtctgtctccag	NM_134455.1
	CCL2	agcatccacgtgctgtctc	gatcatcttgcagtggaatgag	NM_031530.1
	CCL3	gcgctctggaacgaagtct	gaatttgcctccataggag	NM_013025.2
	CCL20	ggggtagctgtggttacct	ggcagcagtcacaaagtgtct	NM_019233.1
	GM-CSF	catctctaagtgttctccacag	cccgtagaccctgtgtgat	NM_053852.1
	VEGF-A	ctatgtgctgcttgggt	gagatagagtatacttcaagccg	NM_001287108.1
	IL6	tggctcttgcgagttccg	agatatgaactagggttccagtattg	NM_012589.2
Interleukins	Calponin	cagccagcgtggcatga	ccatctgcacagctgacg	NM_031747.1
	SM22- α	tatggcagcagtgacagag	cttcttcataaacagcttggga	NM_031549.2
	Smoothelin	gcagtatgaagactacattaccattg	tcagttctgtctctgttg	NM_001013049.2
	SM-MHC	tgagaggagaagaatggctca	tgtagtttctgtctggcagctt	NM_001170600.1
	PRKACA	cggggtctctcatcaga	tgaagtggatgggaacc	NM_001100922.1
PKA subunits	PRKACB	gagtagctggccccagagat	ccaccagtccactgccttat	NM_001077645.1
	PRKAR1A	caataatgaatgggcaacca	atcaaacgccagctctccaaa	NM_013181.1
	PRKAR1B	gatcccccaacgaggagat	gcagggcaatctctccaa	NM_001033679.1
	PRKAR2A	tggatgtgatcggggaaa	aagctgtcggcctttca	NM_019264.2
	PRKAR2B	gtgctctgtggggttg	ttcctcttttggcattgttt	NM_001030020.1
	Cyclophilin A	tgctggaccaaacacaaatg	cttcccaagaccacatgct	NM_017101.1

culture slides with collagen by incubation overnight. The wells were then seeded with cells at a density of 5000 cells/cm², in DMEM containing 1 g/l glucose, 4 mM L-glutamine, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and the plates were incubated for 96 h, at 37 °C, under an atmosphere containing 5% CO₂. The cells were starved by incubation in serum-free DMEM for 7 to 16 h before treatment. We prepared conditioned medium for FRET experiments, by starving the cells in serum-free Fluorobrite DMEM, to minimize background fluorescence.

2.3. Transfection with a small interfering RNA (siRNA)

VSMCs were grown to about 80% confluence, and were maintained for 2 h in antibiotic-free DMEM containing 1 g/l glucose, 4 mM L-glutamine, 10% FCS. Transfection was then performed by adding 0.25% Lipofectamine and 1 nM siRNA in 12.5% Opti-MEM and incubating the cells for 24 h. The cells were then starved of serum and treated for 48 h. The siRNAs used were a mixture of 30 different sequences called siPools purchased from Sitools Biotech GmbH (Planegg, Germany) and

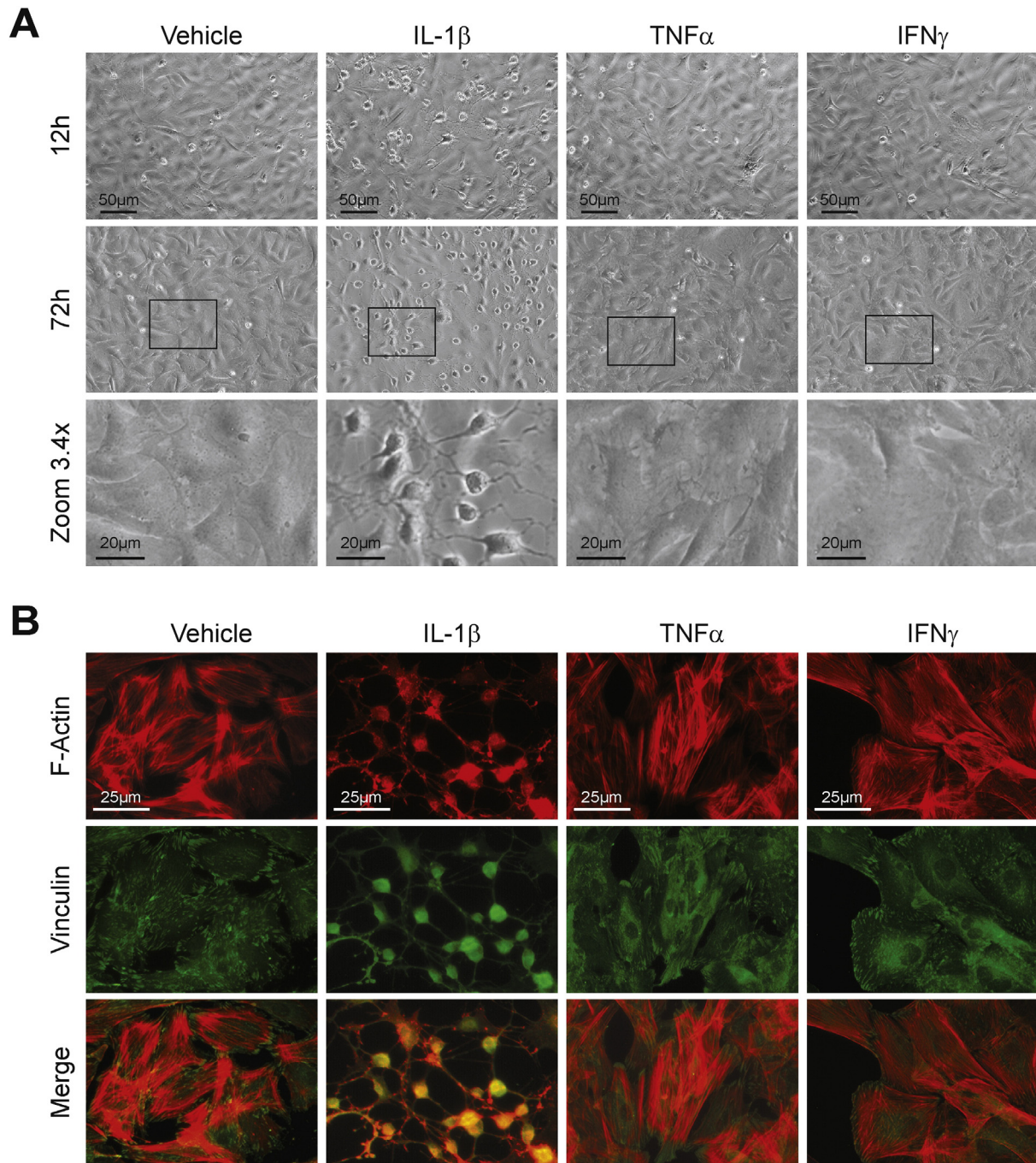


Fig. 1. Effect of pro-atherogenic cytokines on VSMC morphology as a function of cytoskeletal organization. A. Phase-contrast microscopy of cell morphology 12, and 72 h after stimulation with 5 ng/ml IL-1β, 10 ng/ml TNFα or 10 ng/ml IFNγ. B. Immunostaining for filamentous actin (F-actin) and vinculin 72 h after the treatment of PFA-fixed VSMCs, with Alexa Fluor 594-conjugated phalloidin (red) and FITC-coupled monoclonal anti-vinculin antibody (green) visualized by fluorescence microscopy. The images shown are representative of at least 3 independent experiments.

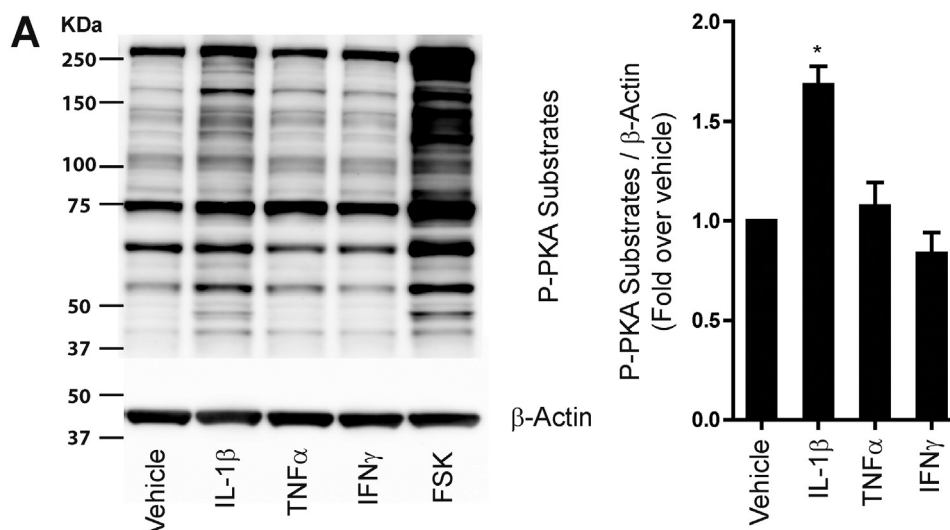


Fig. 2. Effect of pro-atherogenic cytokines on the phosphorylation level of PKA substrates. A. Western-blot analysis of PKA substrate phosphorylation with β -actin as a loading control (left panel). Western-blot quantification for phosphorylated PKA substrates (right panel). The data shown are the means $\pm$ SEM of 5 independent experiments. *, $P < 0.05$, versus vehicle-treated cells in a Kruskal-Wallis test followed by Dunn's multiple comparison test.

directed against rat PRKAR1 α (NM_013181.1) and rat PRKAR1 β (NM_001033679.1).

2.4. Protein extraction and Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS) and scraped into lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Halt phosphatase and protease inhibitor cocktail (Pierce, Thermo-Scientific, Villebon sur Yvette, France). Cell lysates were incubated on a rotating wheel for 15 min at 4 °C. The lysates were then cleared by centrifugation (13,000 $\times$ g for 15 min at 4 °C), and the supernatant was collected. Cell extracts (30 μ g of protein) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4–12% gradient gels (Invitrogen NuPAGE system) and the resulting bands were transferred onto nitrocellulose membranes (0.45 μ m). The membranes were blocked by incubation for 1 h at room temperature with Tris-buffered saline supplemented with 0.1% Tween 20 (TBS-T: 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) and 5% bovine serum albumin, and then incubated overnight with primary antibodies (1/1000) in TBS-T supplemented with 5% bovine serum albumin, at 4 °C. The membranes were then washed in TBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Signals were detected with the ECL detection system, following the exposure of Fujifilm LAS-300, Fujifilm Medical Systems, Stamford, CT, USA. We used β -actin detection to check for equal protein loading and transfer efficiency. The densitometry data obtained were analyzed with ImageJ software and normalized relative to the density of β -actin.

2.5. Absolute quantification of mRNA copy number

Specific 250 to 1000 bp amplicons were generated from the RNA by RT-PCR and purified. Each amplicon was assayed and the number of copy was calculated according to its size in bp. Serial dilutions of known concentration were used in qPCR, to determine the fit coefficients of the relative standard curve. Absolute quantification was performed by interpolation of the PCR signal (Cq) into this standard curve with the Livak-Schmittgen equation [23]. The forward and reverse primers used were as follows: PKAC α , cccgtctctgtgcaaacctg,

ggggtgtggagtgatgta; PKAC β , cgaagtggagagcgtgaaag, gtcttgatgtcactcagcc; PKAR1 α , gcgctgaggtttacactgag, tctcagcggacagtgacac; PKAR1 β , cacactgaggaaacgaaga, catagggatgggtcacagg; PKAR2 α , caataccccgagagctgcta, gaagcaaatggaaggtggg; PKAR2 β , ggtcaaagaagggaacacg, taactgtctcactaacgcgc.

2.6. Quantitative PCR assay

Total RNA was extracted with the RNeasyprep™ RNA isolation kit from Promega, Charbonnières, France. Total RNA (0.5–1 μ g) was reverse-transcribed with the M-MLV reverse transcriptase system and 1 μ M Oligo-dT (Invitrogen, Saint Aubin, France). We carried out qPCR with primers designed with the Roche Universal Probe Library Assay Design Center, with a LightCycler® 480SYBR Green I Master system (Roche Diagnostics, Meylan, France). Cyclophilin A (CycloA) was used as the housekeeping gene. PCR was performed with the following thermal settings: denaturation and enzyme activation at 95 °C for 5 min, followed by variable numbers of cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 10 s. Amplification was monitored online and the reaction was stopped at the end of the logarithmic phase. Melting curve analysis was carried out to check the specificity of PCR. Controls and water blanks were included in each run, and all gave negative results. The qPCR analysis of serial dilutions of a mixture of cDNAs indicated that amplification efficiency was close to 100% for all the genes studied. The primer sequences used to amplify the cDNAs are shown in Table 1.

2.7. Cytokine array

Conditioned medium from cultures of VSMCs treated with vehicle, IL-1 β (5 ng/ml), TNF α (10 ng/ml) or IFN γ (10 ng/ml) for 72 h was incubated with Proteome Profiler Rat Cytokine Array Panel A membranes (R&D Systems, Minneapolis, Minnesota, USA). These concentrations of cytokines were used throughout the study.

2.8. Immunocytochemistry

Collagen-coated coverslips were seeded with 5000 cells/cm². The cells were grown to confluence and starved of serum, and they were

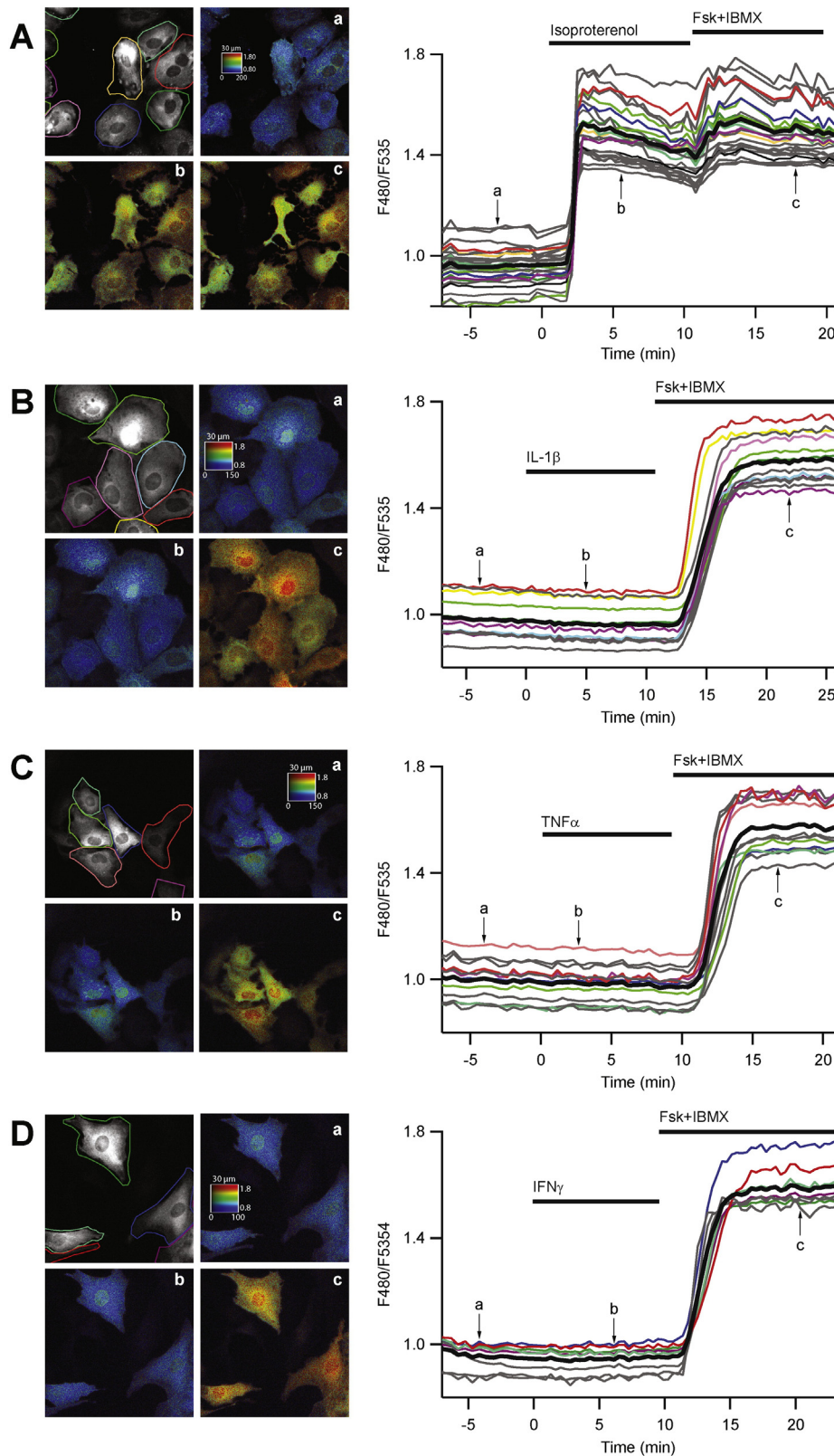


Fig. 3. IL-1 β , TNF α and IFN γ do not increase intracellular cAMP concentration in an acute manner. Changes in intracellular [cAMP] were measured with the $^{\text{T}}\text{Epac}^{\text{V}}$ biosensor, by wide-field fluorescence imaging on untreated VSMCs. Drugs were added to the bath for the period indicated by the bar on the graph: 10 μM isoproterenol (positive control, A), 5 ng/ml IL-1 β (B), 10 ng/ml TNF α (C) or 10 ng/ml IFN γ (D). Left panels. Microscopy fields shown in black and white (upper left) are representative of fluorescence emission at 535 nm. The ratio was determined over individual cells within regions of interest (ROI) delimited with colored contours. The F480/F535 ratio, indicative of biosensor activation, was calculated for each pixel in the whole image. Pseudocolored images representing the F480/F535 emission ratio provide an indication of intracellular [cAMP] (a) before treatment, (b) during cytokines treatments and (c), with 10 μM forskolin + 200 μM IBMX, to maximize biosensor ratio levels. The calibration square (top right) indicates fluorescence intensity (in counts/pixel/s) horizontally and F480/F535 ratio vertically. Right panels. Time course of F480/F535 ratio measurements averaged over individual ROIs. Each trace indicates F480/F535 emission ratio measurements for individual cells over time, corresponding to the ROI delimiting each cell. Gray traces correspond to the F480/F535 emission ratio of cells outside the displayed region. The average of all traces is plotted as a thick black line. The data and images shown are representative of 4 independent experiments.

then treated for 72 h with recombinant IL-1 β , TNF α or IFN γ . Cells were fixed by incubation in 2% sucrose and 3% paraformaldehyde in PBS for 15 min at room temperature, and permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min. Non-specific sites were blocked by incubation with 5% FCS in PBS. The cells were stained for actin and vinculin, by incubation for 2 h at 21 °C with Alexa Fluor® 594-conjugated phalloidin and an FITC-conjugated monoclonal anti-vinculin antibody, in PBS containing 5% FCS. The cell nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI). The coverslips were mounted in Dako fluorescence mounting medium (Dako, Carpinteria, California, USA) and examined with a Nikon Diaphot 300 microscope (Nikon, Tokyo, Japan).

2.9. PGE₂ assay

PGE₂ secretion was evaluated with an enzyme immunoassay kit from Cayman Chemical SPI-BIO, Massy, France.

2.10. Generation of ^TEpac^{vv} cAMP biosensor-encoding adenovirus

^TEpac^{vv}, a FRET-based sensor for cAMP, consists of a cAMP-binding domain from the exchange protein directly activated by cAMP 1 (Epac1) sandwiched between the fluorescent donor mTurquoise and acceptor cpVenus-Venus proteins [24]. The cDNA encoding the ^TEpac^{vv} biosensor was inserted between the *Bgl*II and *Xho*I restriction sites of the pShuttle plasmid (Agilent Biotechnology, Santa Clara, California, USA). The pShuttle-^TEpac^{vv} was linearized by digestion with *Pme*I and inserted into the pAdEasy-1 vector by homologous recombination. Recombinant viral vectors carrying the ^TEpac^{vv} biosensor were produced in the HEK-293 T cell line at UMR1089, Nantes, France.

2.11. Dynamics of cAMP production

VSMCs on collagen-coated glass coverslips were incubated with the ^TEpac^{vv}-encoding adenoviral vector (~100 particles per cell) for 24 h at 37 °C, under an atmosphere containing 5% CO₂. Coverslips were placed in a microscope chamber, continuously perfused (2 ml/min) with a buffer containing 125 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 25 mM glucose and maintained at 32 °C, saturated with 5% CO₂–95% O₂. TNF α , IFN γ or IL-1 β was added during the perfusion.

Conditioned medium for the FRET experiments was obtained by treating VSMCs with IL-1 β , TNF α or IFN γ for 72 h in FluoroBrite™ DMEM, a medium specially developed for the fluorescence imaging of live cells. We measured cAMP production in response to the addition of conditioned medium, in cells that had previously undergone transdifferentiation in response to exposure to IL-1 β , TNF α or IFN γ for 48 h and had then been starved overnight. Ratiometric analyses were performed as follows: fluorescence was induced at an excitation wavelength of 435 nm, and the fluorescence emitted by the donor and acceptor was monitored with a dichroic mirror (455 DCLP) and alternating emission filters for donor (470 ± 20 nm) and acceptor (530 ± 25 nm). Pairs of images were recorded with a CCD camera (Orca-ER, Hammamatus), at 20-s intervals. Changes in intracellular [cAMP] are expressed as the ratio of donor fluorescence (F480nm) to acceptor fluorescence (F535nm). The ratios were multiplied by a constant such that the baseline ratio was 1 in basal conditions. The maximum ratio change (R_{max}) was obtained by stimulating cells with 10 μ M FSK and 200 μ M IBMX. The data shown are representative of three independent experiments, with the acquisition of 10–15 cells per experiment.

2.12. Time-lapse video microscopy

Sixteen hours before any treatment, cells were starved in serum-free DMEM. Changes in their morphology were then monitored by time-lapse video microscopy. Phase-contrast images (3 fields/well) were

captured with a 20 $\times$ objective, at 2.5-minute intervals, for 18 h. Images were captured with an inverted microscope (DMI 6000B from Leica) equipped with a cell incubation chamber, a motorized stage, and a coolSNAP HQ2 CCD camera. All operations were controlled with Metamorph software (version 7.1).

2.13. Statistics

All data are presented as means $\pm$ SEM of at least three independent experiments. Graphical representations were generated and the statistical significance of differences between groups were assessed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Pairwise comparisons of groups were carried out with two-tailed Mann–Whitney tests. For comparisons of multiple groups, we used Kruskal–Wallis tests followed by Dunn's multiple comparison tests. Differences were considered significant if $P < 0.05$.

3. Results

3.1. IL-1 β increases intracellular [cAMP] via one or several autocrine/paracrine factors

Light microscopy (Fig. 1A and Supplementary Videos 1–4) showed that IL-1 β was the only cytokine of those tested capable of inducing the transition to a stellate form in VSMCs. *In vitro*, this phenomenon peaks about 12 h after stimulation, tending to decrease thereafter, as shown by time-lapse imaging. It is followed by a second wave of transition to a stellate morphology beginning 48 h after the addition of IL-1 β and intensifying until 72 h after the addition of this cytokine. The cAMP-dependent stellate morphology of VSMCs (induced by the β -adrenergic receptor agonist isoproterenol) is characterized by a loss of focal adhesions and the disassembly of actin stress fibers [20]. We therefore also stained cells for actin and the focal adhesion marker vinculin. IL-1 β disrupts focal adhesions and actin stress fibers, whereas neither TNF α nor IFN γ modifies the cytoskeleton (Fig. 1B). However, the treatment of VSMCs with IL-1 β , TNF α or IFN γ for 48 h, followed by an analysis of mRNA levels for four major contractile markers showed that all these cytokines inhibited the expression of at least some of these markers, attesting to their efficacy. All three compounds decreased the levels of SM-MHC and calponin (Supplementary Fig. S2), but only IL-1 β and TNF α decreased SM22 (by ~90 and 50%, respectively) and smoothelin (by ~75 and 50%, respectively) mRNA levels to any great extent. Consistent with a cAMP-dependent phenomenon, stellate cells were also obtained following an increase in intracellular [cAMP] induced by forskolin (FSK), a specific activator of adenylyl cyclases (ACs). This effect was observed within a few minutes of FSK application and was maintained for up to 4 h (Supplementary Fig. 1B and Supplementary Video 5). Together with the demonstration that IL-1 β was the only cytokine of those tested to increase PKA activity significantly (as shown by measuring the phosphorylation of PKA substrates, Fig. 2), these data demonstrate that IL-1 β -induced VSMC transdifferentiation is different from that induced by the other cytokines tested. These data also suggest that this effect is at least partly dependent on the cAMP signaling pathway.

We investigated whether the IL-1 β -dependent transdifferentiation of VSMCs was associated with an increase in intracellular cAMP concentration, using the FRET biosensor ^TEpac^{vv} [21]. We monitored changes in intracellular cAMP concentration by calculating the ratio of donor fluorescence (F480nm) to acceptor (F535nm) fluorescence. The baseline ratio at the start of the experiment was set to 1 and the maximum ratio change (R_{max}) was systematically determined at the end of the experiment, by adding 10 μ M forskolin, an activator of adenylyl cyclases, and 200 μ M isobutyl-L-methylxanthine (IBMX), a broad-spectrum phosphodiesterase inhibitor. Incubation with isoproterenol (used as a positive control) greatly increased the ratio to $90.0 \pm 1.1\%$ of the maximal ratio change ($n = 4$, $P < 0.001$), but none of the cytokines tested induced a detectable increase in intracellular

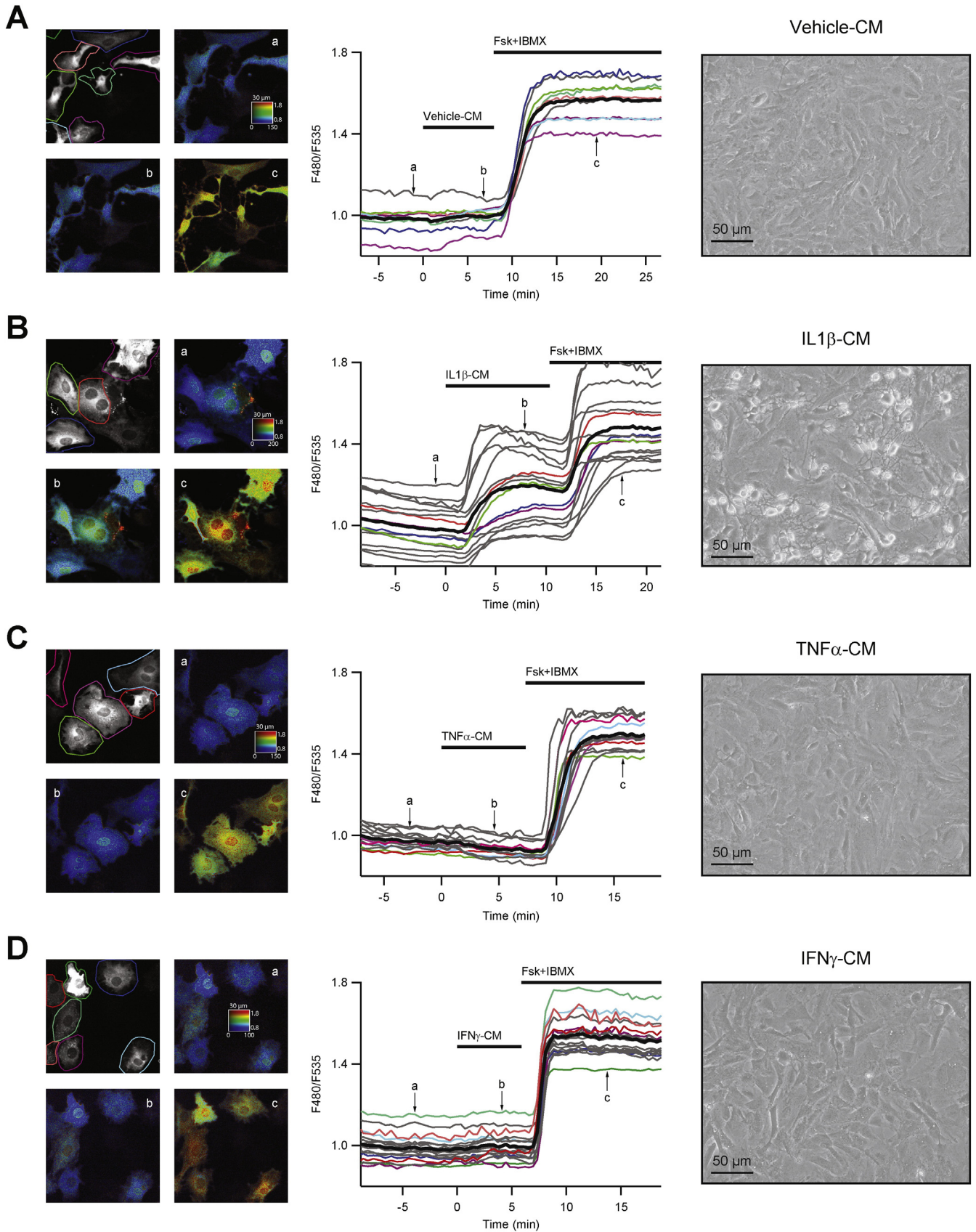


Fig. 4. Conditioned medium (CM) from IL-1 β -treated cells increases cAMP concentration and induces a change in VSMC morphology. CM was collected after the treatment of VSMCs with vehicle, IL-1 β (5 ng/ml), TNF α (10 ng/ml) or IFN γ (10 ng/ml) for 72 h. Recipient cells were allowed to undergo transdifferentiation under the same treatment conditions for 48 h and were then starved overnight. The data and images shown are representative of at least 3 independent experiments. Left and middle panels. Vehicle- (A), IL-1 β - (B), TNF α - (C) or IFN γ -treated VSMCs (D) were perfused with the corresponding conditioned medium for the time indicated by the horizontal bar on the graphs. Left panels. See Fig. 3, legend for the left panel. Middle panels. See Fig. 3, legend for the right panel. Right panels. Phase-contrast microscopy of the morphological phenotype of VSMCs induced by 1 h of treatment with vehicle-CM, IL-1 β -CM, TNF α -CM and IFN γ -CM.

[cAMP] over a similar timeframe (Fig. 3). Given the time taken for IL-1 β to induce the transition to a stellate cell morphology, we hypothesized that any increase in cAMP concentration might be dependent on the production of autocrine/paracrine factors.

We used conditioned medium obtained from VSMCs treated with vehicle, IL-1 β , TNF α or IFN γ for 72 h (referred to as vehicle-CM, IL-1 β -CM, TNF α -CM and IFN γ -CM, respectively) to test this hypothesis. IL-1 β -CM immediately triggered a large increase in intracellular cAMP concentration (up to $34.5 \pm 3.8\%$ of the maximum ratio, $n = 4$, $P < 0.01$), whereas the other conditioned media had no effect on cAMP (Fig. 4 (left and middle panels) and Table 2). Consistent with this finding, the addition of IL-1 β -CM added to naïve/contractile cells also rapidly (within less than 2 h) triggered the transition to a stellate cell morphology (Fig. 4B, right panels), whereas treatment with IL-1 β took at least 12 h to achieve the same effect (Fig. 1A). The other two conditioned media (TNF α -CM and IFN γ -CM) were inefficient regardless of the mode of monitoring used (dynamics of cAMP production or change in cell shape). Thus, stimulation with IL-1 β leads to the release of one or several autocrine/paracrine factors, triggering an increase in the intracellular concentration of cAMP. These findings also demonstrate that cAMP production is a hallmark of IL-1 β -dependent VSMC transdifferentiation.

3.2. The IL-1 β -dependent increase in intracellular [cAMP] involves PGE₂ secretion and PGE₂ receptor isoform 4 (EP4)

As a first step towards identifying the factors involved, we compared the secretion profiles of IL-1 β , TNF α and IFN γ , in terms of their induction of the inflammatory cytokines/chemokines and enzymes involved in eicosanoid/PGE₂ synthesis at the mRNA and/or protein level. For most of the mRNAs studied, significant induction was observed with at least two of the cytokines tested, IL-1 β and TNF α , or IFN γ and TNF α , indicating a very low likelihood of a role in stellate cell morphology for these mRNAs (Table 3 and Fig. 5 left panels A and B). However, the mRNAs encoding COX-2, CCL3, and VEGF-A were specifically and significantly induced by IL-1 β . It was also clear from our results that i) TNF α was the most potent cytokine for CCL5 mRNA synthesis; ii) IFN γ , which had no effect on the CCL5, CCL3, COX-2 and VEGF-A mRNAs, strongly induced synthesis of the CXCL10 mRNA encoding the chemo-attractant of T lymphocytes and monocytes. Using a cytokine array allowing the detection of 29 different inflammatory mediators and a PGE₂ assay, we were able to expand our results further, to show that, in addition to triggering the expression of CCL3 and VEGF, IL-1 β also induced high levels of CXCL2, GM-CSF and IL6 expression, and PGE₂ secretion. It is also clear that i) IFN γ induces the production of small amounts of CXCL1, CX3CL1 and CCL5; ii) contractile vehicle-treated VSMCs have very low levels of secretory activity, because only CXCL1 and the tissue inhibitor matrix metalloproteinase-1 are detected in their culture medium.

Our data and those obtained for human VSMCs [25] suggested that PGE₂ was a potentially good candidate for relaying the increase in cAMP concentration in response to IL-1 β , particularly as PGE₂ triggered the transition to a stellate VSMC morphology within an hour, or even faster in some cases, depending on the number of cell subcultures (Supplementary

Video 6). We therefore repeated the experiments carried out with IL-1 β -CM, successively blocking each of the three PGE₂ receptors expressed by VSMCs.¹ Each dose of antagonist was selected on the basis of published data, to yield maximal inhibition corresponding to 20 times the Ki value [26–28]. Neither the EP1 antagonist nor the EP3 antagonist (SC51322, L798,106, respectively) affected the increase in intracellular [cAMP] induced by IL-1 β -CM (Fig. 6A and C). Conversely, when the EP4 antagonist ONO AE3208 was applied, intracellular [cAMP] rapidly (within 2 min) returned to baseline levels. Consistently with the observation that the transition to a stellate shape was associated with an increase in intracellular [cAMP], ONO AE3 was the only compound capable of blocking this process efficiently in IL-1 β -CM- or PGE₂-treated cells. In light of these findings and the observation that very similar results were obtained when IL-1 β -CM was replaced with PGE₂ (Fig. 6B and D), we conclude that the autocrine/paracrine factor secreted upon IL-1 β stimulation in VSMCs is unique and can be identified as PGE₂.

3.3. The transition to a stellate morphology in VSMCs involves PKA

The regulation of cellular properties by cAMP is mediated principally by cyclic nucleotide-gated ion channels, the exchange proteins directly activated by cAMP (Epac-1 and -2)² and/or protein kinase A (PKA). The treatment of naïve/contractile VSMCs with increasing doses of 6BNZ (a specific activator of PKA) led to a dose-dependent increase in the number of stellate cells (Fig. 7A); 8-pCPT-O-Me-cAMP, an activator of Epac, was ineffective up to a dose of 500 μ M. The specificity of 6BNZ was confirmed by the prevention of its effects on cell morphology at a concentration of 250 μ M by the prior treatment of cells with various doses (100–500 μ M) of Rp-8-Br-cAMP (Supplementary Fig. S3) or Rp-8-CPT-cAMP (data not shown), both of which inhibit PKA. The treatment of VSMCs allowed to transdifferentiate in the presence of IL-1 β (5 ng/ml for 48 h) with 100 μ M 6BNZ for 3 h greatly increased the number of stellate cells (Fig. 7B). By contrast, the same concentration of 6BNZ had almost no effect on contractile/naïve cells (Fig. 7A). Epac stimulation of VSMCs transdifferentiating in response to IL-1 β had no effect, regardless of the batch of 8-pCPT-O-Me-cAMP used (Fig. 7B). These results suggest that the IL-1 β -induced/cAMP-dependent change in shape of VSMCs is mediated by PKA activation.

PKA is a holoenzyme with four subunits, two of which are catalytic, the other two being regulatory. The association of regulatory subunits R1 α /R1 β with any of the catalytic subunits (C α , C β or C γ) defines PKA1, whereas an association of the regulatory subunits R2 α /R2 β with any of the catalytic subunits results in PKA2 and an inhibition of catalytic activity [29]. These two enzymes also differ in terms of their location and affinity for cAMP. Rodents lack the catalytic subunit C γ . A comparison of mRNA levels for each of the subunits between naïve/contractile and IL-1 β -treated VSMCs showed that the contractile/naïve VSMCs produced all four regulatory (R) subunits, the most abundant being R1 α (~65%), followed by R2 α (~22%), R2 β (~10%) and R1 β (~3%); C α and C β accounted for ~70% and 30% of catalytic subunits, respectively (Fig. 7C, left panel). IL-1 β treatment induces a significant 40-fold increase in R1 β gene expression 48 h after stimulation (Fig. 7C, left panel), but R1 α expression nevertheless predominated. The levels of R2 α and R2 β mRNA were affected much less, if at all, by IL-1 β . An analysis of catalytic subunit expression during the process of

Table 2

Amplitude of the dynamic cAMP response triggered by cytokine-treated VSMC conditioned medium. The amplitude of the response is indicated by the mean for the % of the maximum response (Rmax) obtained adding 10 μ M forskolin + 200 μ M IBMX. The data presented are the means $\pm$ SEM of 4 independent experiments on 10–30 individual cells per condition. Significance was evaluated in Kruskal–Wallis tests followed by Dunn's multiple comparison tests. **, $P < 0.01$ versus vehicle-treated cells.

Conditioned medium (CM)	Mean (% of Rmax) $\pm$ SEM
Vehicle-CM	1.39 $\pm$ 0.76
IL-1 β -CM	34.5 $\pm$ 3.8**
TNF α -CM	3.80 $\pm$ 2.4 n.s
IFN γ -CM	2.37 $\pm$ 1.27 n.s

¹ Type E₂ prostaglandin may activate neighboring cells through four different G protein-coupled receptor subtypes, EP1–4. Each of these receptor subtypes is encoded by a different gene and has unique pharmacological features. Contractile and fully secretory rat VSMCs (obtained by incubation with 10 ng/ml IL-1 β , 72 h) express the same pattern of PGE₂ receptor subtype transcript production: 1, 3, 4, but not 2 (Purdy and Arendshorst, 2000; Clément et al., 2006).

² Epacs are GDP/GTP exchange factors for small G proteins of the Ras family, including Rap1A and B, Rap2A and B, and Hras (Schmidt, M., F.J. Dekker, and H. Maarsingh, Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. Pharmacol Rev., 2013. 65(2): p. 670–709).

Table 3

Levels of mRNAs encoding inflammatory markers in transdifferentiated VSMCs. Confluent rat VSMCs were serum-starved and treated with vehicle, IL-1 β (5 ng/ml), TNF α (10 ng/ml) or IFN γ (10 ng/ml). Relative levels of mRNAs encoding inflammatory markers, 48 h after stimulation, expressed as a fold-change with respect to vehicle. The results were first normalized with respect to cyclophilin A (PPIA) and the data shown are the mean value $\pm$ SEM of 7 or 8 independent experiments. Differences between groups were evaluated in Kruskal–Wallis tests followed by Dunn's multiple comparison tests. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ versus vehicle-treated cells.

Gene family	Genes	Fold-change with respect to vehicle			
		Vehicle	IL-1 β	TNF α	IFN γ
Eicosanoid metabolism	COX-2	1.32 $\pm$ 0.38	175 $\pm$ 77 ***	1.4 $\pm$ 0.3 n.s	1.5 $\pm$ 0.3 n.s
	sPLA2 IIA	1.30 $\pm$ 0.36	33,579 $\pm$ 6858 ***	4197 $\pm$ 1445 **	405 $\pm$ 271 n.s
	PTGES1	1.09 $\pm$ 0.18	1399 $\pm$ 359 ***	15.18 $\pm$ 3.95 *	3.96 $\pm$ 1.57 n.s
Cytokines/Chemokines	CXCL1	1.63 $\pm$ 0.6	859 $\pm$ 210 ***	63.4 $\pm$ 9.4 *	25.5 $\pm$ 12.4 n.s
	CXCL2	1.95 $\pm$ 0.89	17,862 $\pm$ 8768 ***	326.2 $\pm$ 130 ***	9.18 $\pm$ 2.88 n.s
	CXCL3	1.08 $\pm$ 0.15	20,027 $\pm$ 7272 ***	546 $\pm$ 98 *	36.5 $\pm$ 23.2 n.s
	CXCL5	2.18 $\pm$ 1.11	18,572 $\pm$ 3777 ***	1162 $\pm$ 204 *	30.24 $\pm$ 9.06 n.s
	CXCL10	1.19 $\pm$ 0.27	9.34 $\pm$ 2.62 n.s	30.48 $\pm$ 3.49 **	5007 $\pm$ 974 ***
	CX3CL1	1.19 $\pm$ 0.22	70.98 $\pm$ 14.72 ***	42.1 $\pm$ 3.45 **	22.24 $\pm$ 6.25 n.s
	CCL2	1.07 $\pm$ 0.17	289 $\pm$ 38 ***	131 $\pm$ 7 **	32.2 $\pm$ 9.8 n.s
	CCL3	1.01 $\pm$ 0.09	1744.36 $\pm$ 632.6 ***	1.30 $\pm$ 0.17 n.s	2.12 $\pm$ 0.75 n.s
	CCL5	1.19 $\pm$ 0.28	3498 $\pm$ 1577 **	21,337 $\pm$ 5183 ***	606 $\pm$ 191 n.s
	CCL20	1.33 $\pm$ 0.53	5915 $\pm$ 1123 ***	569 $\pm$ 105 **	62.44 $\pm$ 38.5 n.s
	GM-CSF	1.02 $\pm$ 0.08	11,029 $\pm$ 2598 ***	67.00 $\pm$ 7.7 *	2.61 $\pm$ 0.81 n.s
	IL6	1.14 $\pm$ 0.18	1043 $\pm$ 143 ***	19.26 $\pm$ 3.16 *	7.32 $\pm$ 2.92 n.s
	VEGF-A	1.10 $\pm$ 0.18	4.02 $\pm$ 0.96 **	1.44 $\pm$ 0.16 n.s	1.53 $\pm$ 0.19 n.s

transdifferentiation revealed no change in the pattern of expression, regardless of the cytokine used (Fig. 7C left panel and data not shown). Based on these data, we decided to silence R1 α to confirm the

involvement of PKA in this process. In many cell types and *in vivo*, the disruption of PKAR1 α is associated with an increase in the catalytic activity of PKA [30–32]. Consistent with a role for PKA in stellate cell

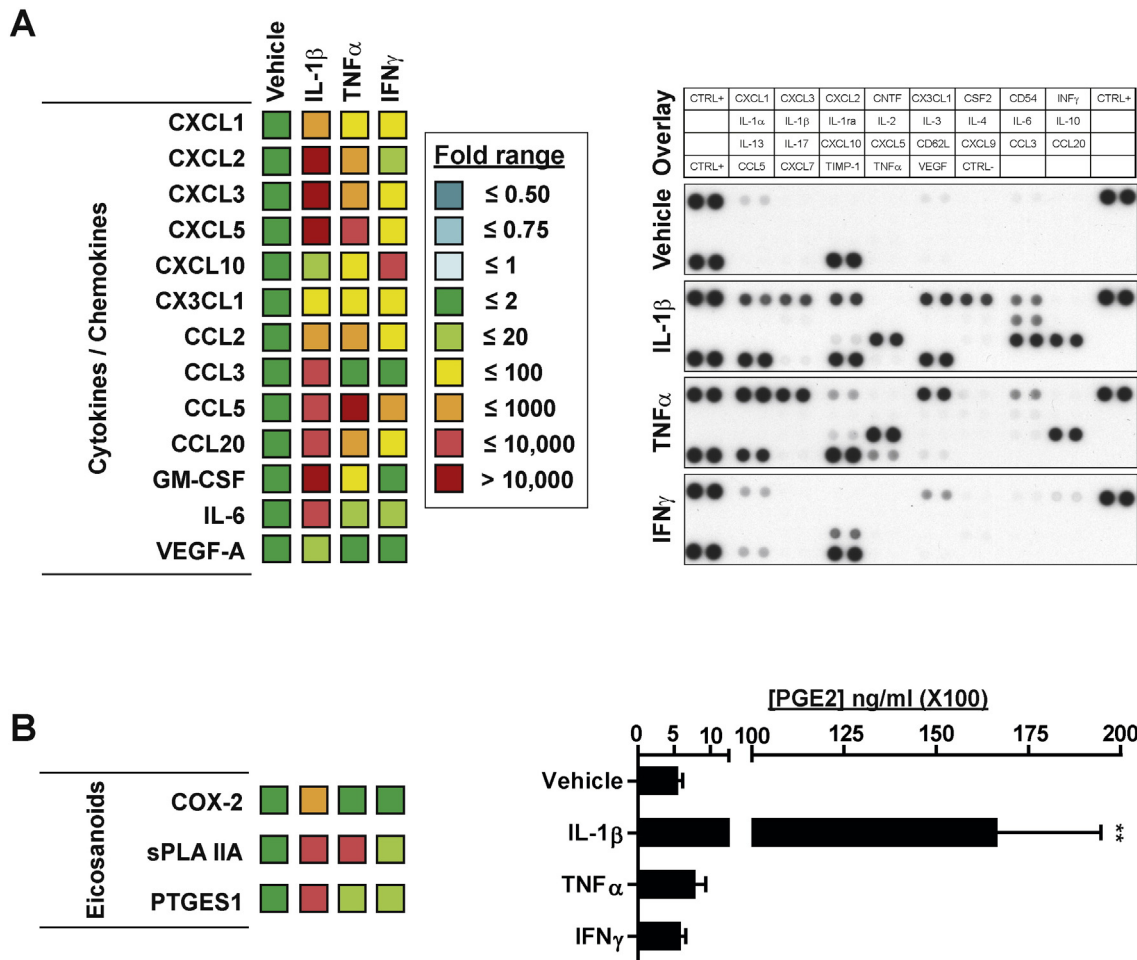


Fig. 5. Inflammatory secretome induced by IL-1 β , TNF α and IFN γ . VSMCs were treated with vehicle, IL-1 β (5 ng/ml), TNF α (10 ng/ml) or IFN γ (10 ng/ml). Left panels A and B. Levels of mRNA after 48 h of stimulation, expressed as fold-changes with respect to vehicle. The diagram is based on the data shown in Table 3 (mean $\pm$ SEM of 8 independent experiments). Right panels A and B. Analysis of the cell medium 72 h after stimulation, in a membrane-based antibody array (A) and by ELISA (B). Data for the PGE₂ assay and inflammatory gene mRNA levels are presented as the mean $\pm$ SEM of 6 and 8 independent experiments respectively. **, $P < 0.01$ versus vehicle-treated cells in Kruskal–Wallis tests followed by Dunn's multiple comparison tests. The results of the cytokine array analysis are representative of 2 independent experiments.

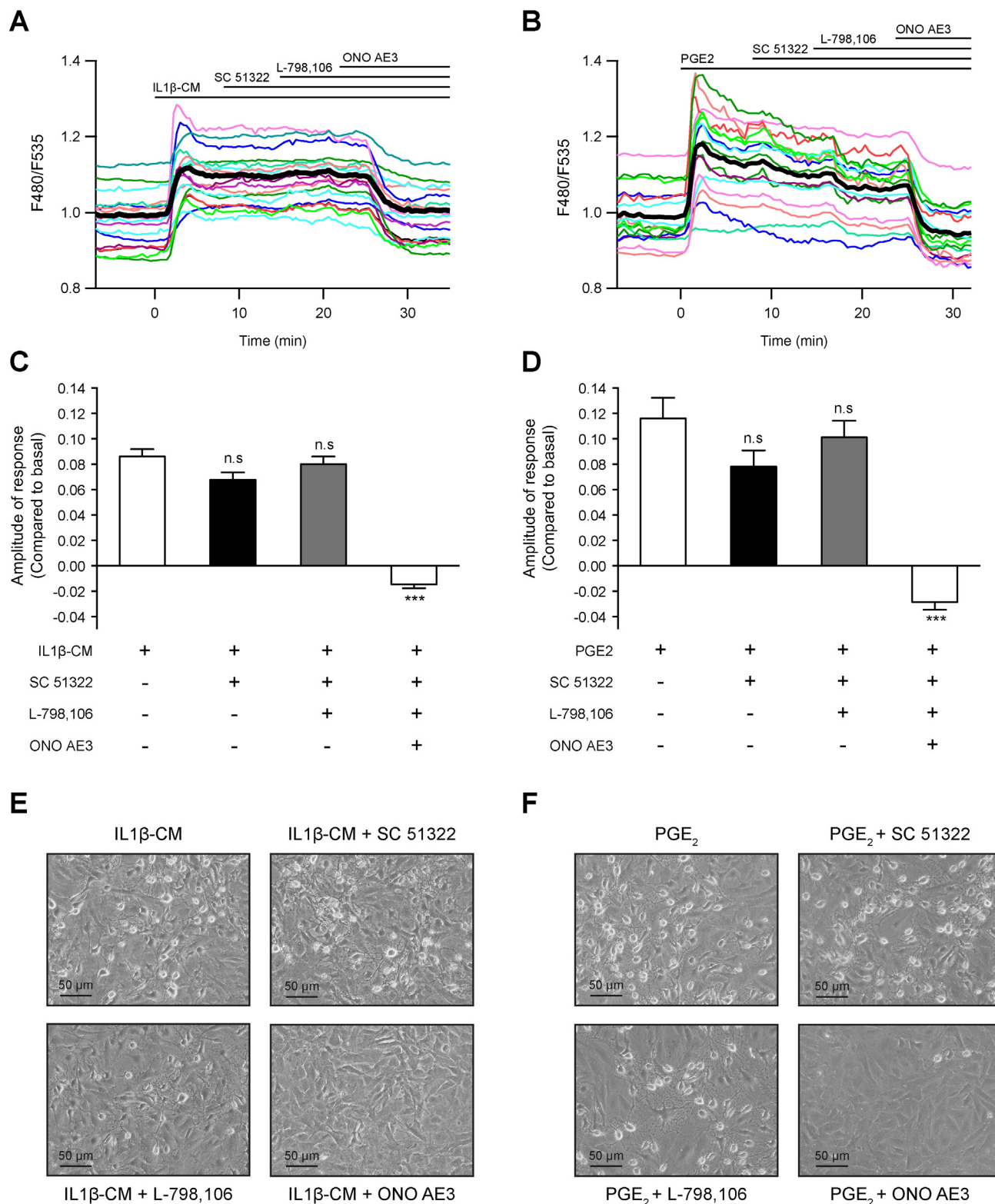


Fig. 6. Blocking EP4 receptors suppresses the response to conditioned medium from IL-1 β -treated VSMCs (IL-1 β -CM, A) and PGE₂ (B). Panels A and B. Intracellular [cAMP] in naive VSMCs treated with (A) IL-1 β -CM or (B) 50 nM PGE₂. The various EP antagonists were added successively, as indicated in the figure. The antagonist concentrations were as follows, 280 nM for SC51322 (EP1), 6 nM for L798,106 (EP3) and 26 nM for ONO AE3208 (EP4). The black line indicates the duration of treatment. See the legend to the right panel in Fig. 3 for details. Panels C and D. Quantification of the changes in cAMP concentration induced by (C) IL-1 β -CM or (D) 50 nM PGE₂ in the presence or absence of the EP antagonists. n.s., non-significant, ***, $P < 0.001$ versus vehicle-treated cells in Kruskal–Wallis tests followed by Dunn's multiple comparison tests. Panels E and F. Phase-contrast microscopy of the changes in VSMC morphology induced by (E) IL-1 β -CM or (F) 50 nM PGE₂ in the presence or absence of the EP antagonists. The data and images shown are representative of 3 independent experiments.

morphology, knocking down R1 α expression mimicked the effect of IL-1 β /PGE₂ on cell morphology, resulting in destabilization of the actin cytoskeleton and focal adhesions (as shown by immunostaining for F-

actin and vinculin, respectively). Conversely, knocking down R1 β expression had no impact (Fig. 7D and Supplementary Fig. S4B). Despite the increase in R1 β mRNA levels observed on IL-1 β treatment, the

silencing of R1 β in PGE₂-treated cells did not prevent these cells from developing a stellate phenotype. The efficacy and specificity of R1 α and β siRNAs were demonstrated by the determination of R1 α and β mRNA levels after transfection (Supplementary Fig. S4A).

4. Discussion

The transdifferentiation of VSMCs in the inflammatory context of atherogenesis plays a key role in atherosclerosis, as highlighted by the recent study by Shankman et al. [15]. Indeed, these authors showed that the contribution of VSMCs to atherosclerotic lesions had been greatly underestimated, due to their ability to develop the expression of macrophage, mesenchymal stem cell and/or myofibroblast markers in lesions. Given the known role of pancreatic stellate cells in acute episode of pancreatic injury [33], it is possible that the stellate cells of the artery wall serve as extracellular matrix-producing cells or myofibroblasts, increasing plaque stability. However, chronic IL-1 β -treatment reduces the expression by VSMCs of profibrotic and/or proproliferative compounds, such as connective tissue growth factor and insulin growth factor-1 (Blirando et al. unpublished data). It therefore appears more likely that the aortic stellate cells first produce large amounts of ECM proteins and then undergo a switch towards a phenotype with high levels of MMP activity. This remains to be demonstrated, but is consistent with the two waves of stellate cell development observed *in vitro* in response to short- and long-term stimulation with IL-1 β .

We show that, of the three key pro-atherogenic cytokines tested (IL-1 β , TNF α and IFN γ), IL-1 β was the most potent inducer (in terms of the numbers and amounts of secreted molecules) of the VSMC inflammatory response, triggering the release of substantial amounts of CCL3, GM-CSF, IL-6, VEGF- α , CX3CL1, CXCL1, CXCL5 and PGE₂. Consistent with published findings, IFN γ was the most efficient of the molecules tested for inducing CXCL10 expression in VSMCs [34] and TNF α was a stronger inducer of CCL5 mRNA production than IL-1 β [35]. Our findings also demonstrate that IL-1 β was the only one of the molecules tested able to trigger the disruption of focal adhesions and actin stress fibers, leading to a cAMP-dependent transition to a stellate morphology, as observed in atherosclerotic lesions [14,20]. Finally, using the FRET-based cAMP biosensor ^{TE}Epac^{vv} and a battery of pharmacological compounds, we were able to link this cAMP-dependent phenomenon to the autocrine/paracrine factor PGE₂, which is released upon IL-1 β stimulation and the activation of PKA by the EP4 prostaglandin receptor.

The IL-1 β -induced increase in intracellular [cAMP] mediated by the release of the autocrine/paracrine factor PGE₂ is consistent with i) the time lag (12 h) observed between the start of IL-1 β -treatment and the development of a cAMP-dependent stellate cell morphology; and ii) our previous results showing that PGE₂ acts in synergy with IL-1 β to enhance the phenotypic transition in smooth muscle cells [22]. It is also consistent with published data showing that the IL-1 β -mediated increase in cAMP concentration in VSMCs is due to the ability of IL-1 β to trigger prostaglandin release [25,36].

Consistent with many other studies showing that EP4 activation leads to an increase in intracellular [cAMP] [37], we showed, by successively blocking each of the EP receptors expressed in VSMCs, that the G_s-coupled EP receptor subtype 4 was involved in the increase in cAMP concentration and the transition to a stellate cell morphology. Following the addition of PGE₂ in conditions of EP receptor subtype 3 blockade, we observed a slight (but non-significant) decrease in cAMP production (Fig. 6B), leading the generation of fewer stellate VSMCs (Fig. 6F, panels PGE₂ vs. PGE₂ + L-798, 106). This finding is consistent with our previous report showing that, in IL-1 β -treated cells, the PGE₂-dependent increase in cAMP concentration results from activation of the EP4 and EP3 receptors [22]. Indeed, transdifferentiation is associated with a change in the regulation (negative to positive) of the Gi-coupled receptor EP3, potentiating the cAMP produced by G_s-coupled EP4 receptors. One reason for the lack of significant results for the EP3 antagonist is that monitoring the dynamics of cAMP production

by FRET requires the removal of IL-1 β from the culture medium 24 h before the experiment. The VSMCs transdifferentiating in response to IL-1 β may, therefore display partial reversion towards a quiescent phenotype, accounting for the small contribution of EP3 to the PGE₂-induced increase in cAMP levels and changes in morphology.

Using the 6BNZ-cAMP (a PKA activator), a siRNA directed against the most abundant PKA regulatory subunit, R1 α , and 8-pCPT-O-Me-cAMP (an Epac activator), we demonstrated the specific involvement of PKA in the cAMP-dependent transition towards a stellate cell phenomenon. However according to Hewer et al. (2011), this transition towards a stellate phenotype in VSMCs results from a synergic activation of PKA and Epac not involving the Epac-dependent Rap1 protein [38]. Several studies have reported Rap1-independent effects of Epac [39,40], but Epac activation generally leads to a Rap1-mediated GDP/GTP exchange [41]. Furthermore, Hewer et al., reported, for 8-pCPT-O-Me-cAMP, a trend towards increasing levels of focal adhesion, a result conflicting with the effects of 8-pCPT-O-Me-cAMP on stellate cell morphology that they report. Thus, for the conclusive implication or exclusion of a role for Epac in this process, further evaluations are required of the possible activation of other downstream Epac effectors, such as the Rit small GTPase or the c-Jun N-terminal kinase, in response to PGE₂ treatment.

As mentioned above, the disruption of PKAR1 α *in vivo* is associated with an increase in the catalytic activity of PKA [30–32], and this increase in catalytic activity (which is lethal in the embryo due to the failure to establish mesoderm-derived structures) is partially rescued by eliminating the PKA catalytic subunit α [42], providing evidence for a causal relationship between the elimination of PKAR1 α and the increase in PKA catalytic activity. Embryonic fibroblasts from PKAR1 α ^{−/−} mouse embryos display similar changes in morphology to VSMCs in which R1 α is silenced, with a decrease in activated Rac levels [32]. Moreover, the transition of VSMCs to a stellate morphology induced by agents increasing cAMP levels, such as isoproterenol and forskolin, can be prevented by the expression of an activated form of Rac1. Conversely, the pharmacological inhibition of Rac1 or the expression of a dominant negative form of Rac1 is sufficient to induce the transition to a stellate phenotype [20]. Finally, PKAR1 α disruption in Schwann cells is associated with a decrease in the formation of lamellopodia [31], a process involving Rac1 activation [43]. This suggests a role for Rac1 in the PGE₂/cAMP-dependent development of stellate morphology in VSMCs. Furthermore, as PKA phosphorylates key actors involved in cytoskeletal regulation, such as the vasodilator-stimulated phosphoprotein (VASP) [18], Ras homolog gene family member A (RhoA) [44] and LIM domain kinase 1 (LIMK1) [32], these proteins may also be involved in the cAMP-dependent transition to a stellate phenotype. In any event, PKA enhances this process by phosphorylating globular actin (G-actin), thereby decreasing filamentous actin (F-actin) formation [44]. Indeed, PKAR1 α silencing induced a destabilization of filamentous actin and the disassembly of focal adhesions (Fig. 7C).

IL-1 β -dependent transdifferentiation is associated with an increase in PKAR1 β gene expression. Nevertheless, the silencing of this subunit does not prevent VSMCs from undergoing the transition to a stellate phenotype following stimulation with PGE₂ (Fig. 7B). We therefore conclude that PKAR1 β is not required for this process.

PKA containing PKAR1 β subunits has been shown to be activated at cAMP concentrations lower, by a factor of three to seven, than those activating PKAR1 α [45]. Therefore PKA-dependent responses should be even more sensitive to any increase in intracellular cAMP concentration in VSMCs undergoing transdifferentiation in response to IL-1 β . Interestingly, in PKAR1 β -knockout mice, inflammation and nociceptive pain levels are low in a model of paw edema and pain behavior induced by formalin injection [46]. In addition, patients with systemic lupus erythematosus present a downregulation of PKAR1 β expression and lower levels of IL-2 secretion by T lymphocytes, these deficits possibly being rescued by PKAR1 β gene transfer [47]. The data presented here are consistent with a role for PKAR1 β in the increase in cAMP concentration mediating VSMC inflammation and, possibly, PGE₂ secretion.

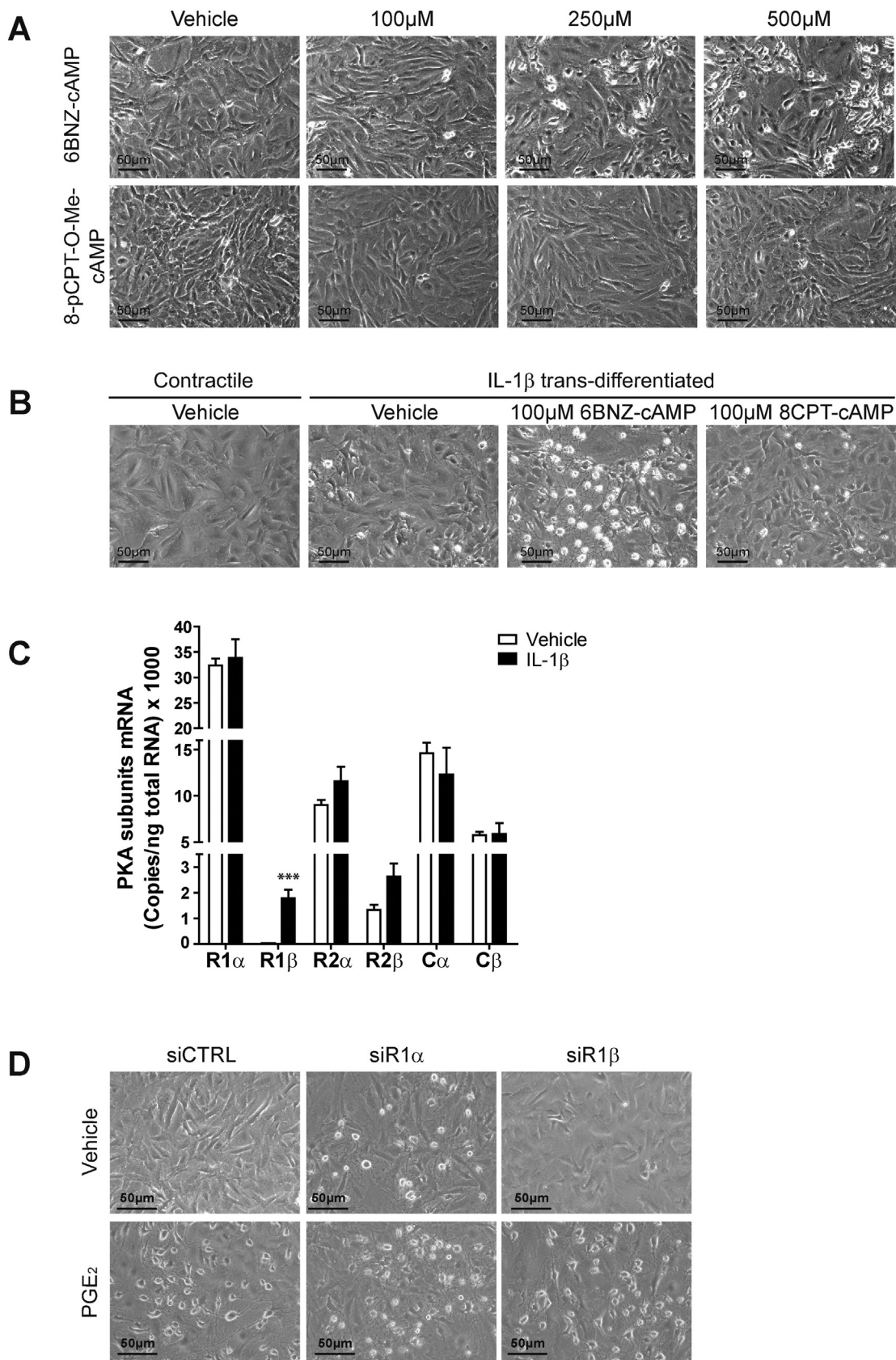


Fig. 7. Role of PKA in the development of a stellate morphology in VSMCs. Phase-contrast microscopy of naive (A) and IL-1 β -transdifferentiated (B) VSMCs in the presence of vehicle, Epac (8-pCPT-O-Me-cAMP, 100 μ M) and/or PKA (6BNZ-cAMP, 100 μ M) activators. The concentrations used for each compound are indicated on the figure. The results shown are representative of at least 3 independent experiments. C. Numbers of copies of PKA regulatory and catalytic subunit mRNAs in VSMCs treated with vehicle or IL-1 β (5 ng/ml) for 48 h. The data shown are the means $\pm$ SEM of at least 4 independent experiments. *, $P < 0.05$, ***, $P < 0.001$ versus vehicle-treated cells in Kruskal–Wallis tests followed by Dunn's multiple comparison test. D. Phase-contrast microscopy of VSMCs transfected with control siRNA (siCTRL) or siRNA directed against the PKAR1 α (siR1 α) or PKAR1 β (siR1 β) subunits. Upper and lower panels: vehicle- and PGE₂-treated cells (50 nM for 1 h), respectively.

5. Conclusions and future prospects

VSMC transdifferentiation in response to exposure to pro-atherogenic stimuli, such as the cytokines IL-1 β , TNF α and IFN γ , is a key event in the initiation and further development of atherosclerosis. We demonstrate here that IL-1 β contributes to this transdifferentiation process not only by decreasing the expression of contractile markers, but also by disrupting F-actin and focal adhesions through the release of PGE₂ and subsequent cAMP pathway activation. Given these and published findings, it will be of interest to investigate the effect on plaque development of VSMC-directed PKAR1 α and/or PKAR1 β gene knockout in atherosclerosis-susceptible mouse models. Indeed, such studies should define the roles of these molecules in the onset of atherosclerosis and their potential relevance as treatment targets. Studies on human atherothrombotic plaques should provide insight into the relationship between PKAR1 α and/or PKAR1 β expression and vulnerability to plaque formation. They should also provide a more general view of the role of this relationship in human atherosclerosis.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamcr.2015.09.019>.

Acknowledgments

We thank Susanne Bolte, Richard Schwartzmann and Jean-François Gilles from the imaging facility of the Paris-Seine Biological Institute (IBPS). This work was supported by Paris VI University and the national research agency, under grant number 11 BSV1 034 01.

References

- [1] P. Libby, P.M. Ridker, G.K. Hansson, Progress and challenges in translating the biology of atherosclerosis, *Nature* 473 (2011) 317–325.
- [2] K. Sakakura, M. Nakano, F. Otsuka, E. Ladich, F.D. Kolodgie, R. Virmani, Pathophysiology of atherosclerosis plaque progression, *Heart Lung Circ.* 22 (2013) 399–411.
- [3] B. Legein, L. Temmerman, E.A. Biessen, E. Lutgens, Inflammation and immune system interactions in atherosclerosis, *Cell. Mol. Life Sci.* 70 (2013) 3847–3869.
- [4] G.K. Hansson, A. Hermansson, The immune system in atherosclerosis, *Nat. Immunol.* 12 (2011) 204–212.
- [5] J.E. McLaren, D.P. Ramji, Interferon gamma: a master regulator of atherosclerosis, *Cytokine Growth Factor Rev.* 20 (2009) 125–135.
- [6] P. Hellstrand, S. Albinsson, Stretch-dependent growth and differentiation in vascular smooth muscle: role of the actin cytoskeleton, *Can. J. Physiol. Pharmacol.* 83 (2005) 869–875.
- [7] C.P. Mack, J.S. Hinson, Regulation of smooth muscle differentiation by the myocardin family of serum response factor co-factors, *J. Thromb. Haemost.* 3 (2005) 1976–1984.
- [8] M.S. Parmacek, Myocardin-related transcription factors: critical coactivators regulating cardiovascular development and adaptation, *Circ. Res.* 100 (2007) 633–644.
- [9] J. Shen, M. Yang, D. Ju, H. Jiang, J.P. Zheng, Z. Xu, L. Li, Disruption of SM22 promotes inflammation after artery injury via nuclear factor kappaB activation, *Circ. Res.* 106 (2010) 1351–1362.
- [10] J. Shen, M. Yang, H. Jiang, D. Ju, J.P. Zheng, Z. Xu, T.D. Liao, L. Li, Arterial injury promotes medial chondrogenesis in Sm22 knockout mice, *Cardiovasc. Res.* 90 (2011) 28–37.
- [11] M.F. Carlier, J. Pernier, P. Montaville, S. Shekhar, S. Kuhn, Control of polarized assembly of actin filaments in cell motility, *Cell. Mol. Life Sci.* (2015).
- [12] S.J. Gunst, W. Zhang, Actin cytoskeletal dynamics in smooth muscle: a new paradigm for the regulation of smooth muscle contraction, *Am. J. Physiol. Cell Physiol.* 295 (2008) C576–C587.
- [13] M.A. Schwartz, R.K. Assoian, Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways, *J. Cell Sci.* 114 (2001) 2553–2560.
- [14] A.N. Orekhov, E.R. Andreeva, A.V. Krushinsky, I.D. Novikov, V.V. Tertov, G.V. Nestaiko, K. Khashimov, V.S. Repin, V.N. Smirnov, Intimal cells and atherosclerosis. Relationship between the number of intimal cells and major manifestations of atherosclerosis in the human aorta, *Am. J. Pathol.* 125 (1986) 402–415.
- [15] L.S. Shankman, D. Gomez, O.A. Cherepanova, M. Salmon, G.F. Alencar, R.M. Haskins, P. Swiatlowski, A.A. Newman, E.S. Greene, A.C. Straub, B. Isakson, G.J. Randolph, G.K. Owens, KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis, *Nat. Med.* 21 (2015) 628–637.
- [16] E. Seki, D.A. Brenner, Recent advancement of molecular mechanisms of liver fibrosis, *J. Hepatobiliary Pancreat. Sci.* 22 (2015) 512–518.
- [17] G.N. Chaldakov, T. Nabika, Y. Nara, Y. Yamori, Cyclic AMP- and cytochalasin B-induced arborization in cultured aortic smooth muscle cells: its cytopharmacological characterization, *Cell Tissue Res.* 255 (1989) 435–442.
- [18] R. Gros, Q. Ding, J. Chorazyczewski, J.G. Pickering, L.E. Limbird, R.D. Feldman, Adenylyl cyclase isoform-selective regulation of vascular smooth muscle proliferation and cytoskeletal reorganization, *Circ. Res.* 99 (2006) 845–852.
- [19] T. Nabika, P.A. Velletri, T. Igawa, Y. Yamori, W. Lovenberg, Comparison of cyclic AMP accumulation and morphological changes induced by beta-adrenergic stimulation of cultured vascular smooth muscle cells and fibroblasts, *Blood Vessels* 22 (1985) 47–56.
- [20] S. Pelletier, C. Julien, M.R. Popoff, N. Lamarche-Vane, S. Meloche, Cyclic AMP induces morphological changes of vascular smooth muscle cells by inhibiting a Rac-dependent signaling pathway, *J. Cell. Physiol.* 204 (2005) 412–422.
- [21] J. Klarenbeek, K. Jalink, Detecting cAMP with an EPAC-based FRET sensor in single living cells, *Methods Mol. Biol.* 1071 (2014) 49–58.
- [22] N. Clement, M. Glorian, M. Raymondjean, M. Andreani, I. Limon, PGE2 amplifies the effects of IL-1beta on vascular smooth muscle cell de-differentiation: a consequence of the versatility of PGE2 receptors 3 due to the emerging expression of adenylyl cyclase 8, *J. Cell. Physiol.* 208 (2006) 495–505.
- [23] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−delta delta C(T)) method, *Methods* 25 (2001) 402–408.
- [24] J.B. Klarenbeek, J. Goedhart, M.A. Hink, T.W. Gadella, K. Jalink, A mTurquoise-based cAMP sensor for both FLIM and ratiometric read-out has improved dynamic range, *PLoS One* 6 (2011), e19170.
- [25] D. Beasley, COX-2 and cytosolic PLA2 mediate IL-1beta-induced cAMP production in human vascular smooth muscle cells, *Am. J. Phys.* 276 (1999) H1369–H1378.
- [26] E.A. Hallinan, A. Stapelfeld, M.A. Savage, M. Reichman, 8-chlorodibenz[b,f][1,4]oxazepine-10(11 H)-carboxylic acid, 2-[3-[2-(furanymethyl)thio]-1 oxopropyl] hydrazide (SC-51322): A potent PGE2 antagonist and analgesic, *Bioorg. Med. Chem. Lett.* 4 (1994) 509–514.
- [27] H. Juteau, Y. Gareau, M. Labelle, C.F. Sturino, N. Sawyer, N. Tremblay, S. Lamontagne, M.C. Carriere, D. Denis, K.M. Metters, Structure-activity relationship of cinnamic acylsulfonamide analogues on the human EP3 prostanoid receptor, *Bioorg. Med. Chem.* 9 (2001) 1977–1984.
- [28] K. Kabashima, T. Saji, T. Murata, M. Nagamachi, T. Matsuoka, E. Segi, K. Tsuboi, Y. Sugimoto, T. Kobayashi, Y. Miyachi, A. Ichikawa, S. Narumiya, The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut, *J. Clin. Invest.* 109 (2002) 883–893.
- [29] S.S. Taylor, R. Ilouz, P. Zhang, A.P. Kornev, Assembly of allosteric macromolecular switches: lessons from PKA, *Nat. Rev. Mol. Cell Biol.* 13 (2012) 646–658.
- [30] X. Cheng, Z. Ji, T. Tsalkova, F. Mei, Epac and PKA: a tale of two intracellular cAMP receptors, *Acta Biochim. Biophys. Sin.* 40 (2008) 651–662.
- [31] L. Guo, A.A. Lee, T.A. Rizvi, N. Ratner, L.S. Kirschner, The protein kinase A regulatory subunit R1A (Prkar1a) plays critical roles in peripheral nerve development, *J. Neurosci.* 33 (2013) 17967–17975.
- [32] K.S. Nadella, M. Saji, N.K. Jacob, E. Pavel, M.D. Ringel, L.S. Kirschner, Regulation of actin function by protein kinase A-mediated phosphorylation of Limk1, *EMBO Rep.* 10 (2009) 599–605.
- [33] J.S. Wilson, R.C. Pirola, M.V. Apte, Stars and stripes in pancreatic cancer: role of stellate cells and stroma in cancer progression, *Front. Physiol.* 5 (2014) 52.
- [34] X. Wang, T.L. Yue, E.H. Ohlstein, C.P. Sung, G.Z. Feuerstein, Interferon-inducible protein-10 involves vascular smooth muscle cell migration, proliferation, and inflammatory response, *J. Biol. Chem.* 271 (1996) 24286–24293.
- [35] N.J. Jordan, M.L. Watson, R.J. Williams, A.G. Roach, T. Yoshimura, J. Westwick, Chemokine production by human vascular smooth muscle cells: modulation by IL-13, *Br. J. Pharmacol.* 122 (1997) 749–757.
- [36] D. Beasley, M.E. McGuiggin, Interleukin 1 induces prostacyclin-dependent increases in cyclic AMP production and does not affect cyclic GMP production in human vascular smooth muscle cells, *Cytokine* 7 (1995) 417–426.
- [37] S. Narumiya, Y. Sugimoto, F. Ushikubi, Prostanoid receptors: structures, properties, and functions, *Physiol. Rev.* 79 (1999) 1193–1226.
- [38] R.C. Hewer, G.B. Sala-Newby, Y.J. Wu, A.C. Newby, M. Bond, PKA and Epac synergistically inhibit smooth muscle cell proliferation, *J. Mol. Cell. Cardiol.* 50 (2011) 87–98.
- [39] D. Hochbaum, T. Tanos, F. Ribeiro-Neto, D. Altschuler, O.A. Coso, Activation of JNK by Epac is independent of its activity as a Rap guanine nucleotide exchanger, *J. Biol. Chem.* 278 (2003) 33738–33746.
- [40] G.X. Shi, H. Rehmann, D.A. Andres, A novel cyclic AMP-dependent Epac-Rit signaling pathway contributes to PACAP38-mediated neuronal differentiation, *Mol. Cell. Biol.* 26 (2006) 9136–9147.
- [41] S.S. Roscioni, C.R. Elzinga, M. Schmidt, Epac: effectors and biological functions, *Naunyn Schmiedeberg's Arch. Pharmacol.* 377 (2008) 345–357.
- [42] L.S. Kirschner, Z. Yin, G.N. Jones, E. Mahoney, Mouse models of altered protein kinase A signaling, *Endocr. Relat. Cancer* 16 (2009) 773–793.
- [43] A.N. Lyle, W.R. Taylor, RACing up a new regulatory mechanism for vascular smooth muscle cell migration, *Arterioscler. Thromb. Vasc. Biol.* 33 (2013) 667–669.
- [44] A.K. Howe, Regulation of actin-based cell migration by cAMP/PKA, *Biochim. Biophys. Acta* 1692 (2004) 159–174.
- [45] G.G. Cadd, M.D. Uhler, G.S. McKnight, Holoenzymes of cAMP-dependent protein kinase containing the neural form of type I regulatory subunit have an increased sensitivity to cyclic nucleotides, *J. Biol. Chem.* 265 (1990) 19502–19506.
- [46] A.B. Malmberg, E.P. Brandon, R.L. Idzerda, H. Liu, G.S. McKnight, A.I. Basbaum, Diminished inflammation and nociceptive pain with preservation of neuropathic pain in mice with a targeted mutation of the type I regulatory subunit of cAMP-dependent protein kinase, *J. Neurosci.* 17 (1997) 7462–7470.
- [47] I.U. Khan, D. Laxminarayana, G.M. Kammer, Protein kinase A RI beta subunit deficiency in lupus T lymphocytes: bypassing a block in RI beta translation reconstitutes protein kinase A activity and augments IL-2 production, *J. Immunol.* 166 (2001) 7600–7605.