Cyclic AMP-Rap1A signaling activates RhoA to induce \( \alpha_{2c} \)-adrenoceptor translocation to the cell surface of microvascular smooth muscle cells

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Jeyaraj SC, Unger NT, Eid AH, Mitra S, El-Dahdah NP, Quilliam LA, Flavahan NA, Chotani MA. Cyclic AMP-Rap1A signaling activates RhoA to induce \( \alpha_{2c} \)-adrenoceptors to the cell surface of microvascular smooth muscle cells. Am J Physiol Cell Physiol 303: C499–C511, 2012. First published May 23, 2012; doi:10.1152/ajpcell.00461.2011.—Intracellular signaling by the second messenger cyclic AMP (cAMP) activates the Ras-related small GTPase Rap1 through the guanine exchange factor Epac. This activation leads to effector protein interactions, activation, and biological responses in the vasculature, including vasorelaxation. In vascular smooth muscle cells derived from human dermal arterioles (micro-VSM), Rap1 selectively regulates expression of \( \alpha_{2c} \)-adrenoceptors (\( \alpha_{2c} \)-ARs) through JNK-c-jun nuclear signaling. The \( \alpha_{2c} \)-ARs are generally retained in the trans-Golgi compartment and mobilize to the cell surface and elicit vasorelaxation in response to cellular stress. The present study used human microVSM to examine the role of Rap1 in receptor localization. Complementary approaches included murine microVSM derived from tail arteries of C57BL/6 mice that express functional \( \alpha_{2c} \)-ARs and mice deficient in Rap1A (Rap1A-null). In human microVSM, increasing intracellular cAMP by direct activation of adenyl cyclase by forskolin (10 \( \mu \)M) or selectively activating Epac-Rap signaling by the cAMP analog 8-pCPT-2′-O-Me-cAMP (100 \( \mu \)M) activated RhoA, increased \( \alpha_{2c} \)-AR expression, and reorganized the actin cytoskeleton, increasing F-actin. The \( \alpha_{2c} \)-ARs mobilized from the perinuclear region to intracellular filamentous structures and to the plasma membrane. Similar results were obtained in murine wild-type microVSM, coupling Rap1-Rho-axon dynamics to receptor relocalization. This signaling was impaired in Rap1A-null murine microVSM and was rescued by delivery of constitutively active (CA) mutant of Rap1A. When tested in heterologous HEK293 cells, Rap1A-CA or Rho-kinase (ROCK-CA) caused translocation of functional \( \alpha_{2c} \)-ARs to the cell surface (4- to 6-fold increase, respectively). Together, these studies support vascular bed-specific physiological role of Rap1 and suggest a role in vasoconstriction in response to cellular stress.

G-protein-coupled receptor; Rap1A-null; actin cytoskeleton; 8-pCPT-2′-O-Me-cAMP

The monomeric GTP-binding protein Rap1 is a molecular switch that relays extracellular stimulation to intracellular signaling upon activation by guanine nucleotide exchange factors, including Epac (exchange protein directly activated by cyclic AMP) that couples to second messenger cyclic AMP signaling (31). Epac1 is the predominant isoform expressed in vascular tissue and in vascular smooth muscle cells (VSMs) (23, 39, 57). In VSMs from large vessels such as aorta and pulmonary artery, intracellular cAMP signaling is generally associated with vasorelaxation (57). Elucidation of signaling pathways shows that cAMP mediates smooth muscle relaxation through activation of A-kinase and Epac-Rap1 signaling, inhibiting RhoA activity and RhoA-mediated calcium-sensitized force (14, 57). The mechanisms involved in this inhibition include direct inactivation of RhoA (14), reduction in intracellular cytoplasmic calcium concentration (44, 50), phosphorylation of the protein telokin that activates myosin light chain phosphatase (MLCP) activity (54), activation of myosin phosphate targeting regulatory subunit (MYPT1), and disinhibition of MLCP activity (14, 52, 54).

VSMs derived from human cutaneous arterioles (micro-VSMs) express both Rap1 and Rap2 subtypes. However, increasing intracellular cAMP by forskolin leads to rapid and transient increase in GTP-bound active Rap1, but not Rap2 (8). Unlike large arteries, cutaneous arterioles (also known as resistance vessels) possess pressure-induced myogenic contractile activity, inherent phasic contractile activity, and express functional \( \alpha_{2c} \)-adrenoceptors (7, 9). Indeed, cutaneous arterioles demonstrate a unique and physiologically important cold-induced contractile response that is mediated by cold-induced amplification of \( \alpha_{2c} \)-adrenoceptor (\( \alpha_{2c} \)-AR) activity (7).

MicroVSM \( \alpha_{2c} \)-ARs are generally retained in the intracellular Golgi compartment (visualized as perinuclear receptors) and mobilized to the cell surface during conditions of stress or in response to cold temperature (7–9, 30). Once on the cell surface, \( \alpha_{2c} \)-ARs can respond to receptor agonists by mediating vasoconstriction, including to the endogenous agonist noradrenaline. In human microVSMs, exogenous expression of Rap1 subtype A (Rap1A) increased transcription of \( \alpha_{2c} \)-ARs via a JNK-c-jun signaling pathway, suggesting a role for Rap1A in vasoconstriction (12). In this study we have examined the role of endogenous Rap1 in \( \alpha_{2c} \)-AR expression and localization by utilizing human microVSMs and Rap1A-null murine microVSMs. We show that Rap1A is not only necessary for endogenous gene expression of \( \alpha_{2c} \)-ARs, but is also necessary for translocation of perinuclear receptors to actin filaments and to the cell surface. We show that the mechanism of receptor mobilization involves activation of Rho and Rho-associated kinase ROCK, which is similar to the mechanism...
identified for cold-triggered signaling and receptor translocation.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**

HA-tagged receptors. The murine amino terminus hemagglutinin (HA)-tagged α2C-ARs have previously been described (11, 30).

Adenovirus-Rap1A-CA and Rap1A-DN. Recombinant adenovirus (Ad) constructs were generated by the method of Vogelstein and colleagues (22). Recombinant vectors included Rap1A-63E (constitutively active; Ad-Rap1A-CA) and Rap1A-17N (dominant inhibitory or negative; Ad-Rap1A-DN; original plasmid DNA in pcDNA3 backbone). The Rap1A fragments were excised by digestion with KpnI-XhoI and insertion in the KpnI-XhoI sites of pShuttle-CMV vector, linearization with Pmel and cotransformation with pAd-Easy1 vector in BJ5183-AD-1 electroporation competent cells (Stratagene, La Jolla, CA). Recombinant adenovirus vectors were generated by following manufacturer protocol (AdEasy XL, Adenoviral Vector System, Stratagene).

Vector-CMV backbone without insert (Ad-Control) was used as control for transductions. Preparation of high titer viral stocks was facilitated by the Viral Vector Core at the Research Institute at Nationwide Children's Hospital, and included adenovirus plaque purifications, amplifications, and large-scale expansions, and titer [in plaque-forming units (pfu)/ml, using the TCD50 titration method] determination to generate high titer virus stocks.

**ROCK.** The constitutively active mutant ROCK-CA plasmid DNA (p160-ROCKA3, carboxyl-terminus truncated ROCK, in the backbone plasmid pCAG-myc) was kindly provided by Dr. Shuh Narumiya (Faculty of Medicine, Kyoto University, Kyoto, Japan) and has been previously described (27). Overexpression of ROCK-CA was detected by an antibody directed against the Myc-tag.

**Antibodies**

1) Anti-α2C-AR affinity purified rabbit polyclonal antibody targeting the carboxy terminus of α2C-adrenoceptors was custom generated (Bethyl Laboratories, Houston, TX). We have established the specificity of this antibody in previous studies (1, 9). This antibody recognizes the three main α2C-AR molecular species, including the 50–55 kDa core glycosylated form, the 70–80 kDa mature form, and the 100–120 kDa dimer of the 50–55 kDa form (6). The 70–80 kDa mature receptor form mobilizes from the trans-Golgi compartment to the cell surface (1, 30), and it is the main form quantitated in this study.

2) Anti-actin, α-smooth muscle (monoclonal, clone 1A4), and anti-calponin (monoclonal, clone hCPC) were purchased from Sigma (St. Louis, MO).

3) Anti-RhoA (mouse monoclonal, clone 55/Rho) was purchased from BD Transduction Laboratories (Lexington, KY).

4) Anti-HA.11 monoclonal antibody (clone 16B12) was purchased from Covance (Berkeley, CA). Anti-Myc (9B11) monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA).

5) Anti-Aequorea victoria green fluorescent protein (GFP) affinity-purified monoclonal antibody (JL-8) was purchased from Clontech (Mountain View, CA).

6) Anti-β-actin (clone AC-15) was purchased from Sigma-Aldrich. Alternatively, anti-glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibody was used, and was purchased from Abcam (Cambridge, MA).

7) Anti-Rap1 antibody was purchased from Millipore (Temecula, CA). The rabbit anti-Rap1B was custom generated; the specificity of this antibody has been established in previous studies (56).

All secondary peroxidase-labeled (anti-mouse and anti-rabbit) antibodies were purchased from GE Healthcare/Amersham (United Kingdom).

**Chemicals**

The adenylyl cyclase activator forskolin, the α2-AR agonist UK14,304, and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma; the membrane-permeant Epac-Rap1 activator 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8-pCPT-2′-O-Me-cAMP) was purchased from BIOLOG Life Science Institute [Bremen, Germany; selective for Epac, with no detectable effect on A-kinase (4)]. Exoenzyme C3 transferase from Clostridium botulinum (Cytoskeleton, Denver, CO) was used to inhibit endogenous Rho activity. Fadusil hydrochloride (1-(5-isquinolinesulfonyl)-homopiperazine hydrochloride, Tocris Bioscience, Ellisville, MO) and H-1152 (5′--(+)--2-2 methyl-1-(4 methyl-5 isquinolinosulfonyl)sulfonil)-homopiperazine, 2HCl) were used to inhibit Rho-kinase, and cytochalasin D (Tocris) was used to disrupt F-actin.

**Human VSM Culture**

Dermal arterial VSMs were cultured from arterioles isolated from upper arm skin punch biopsies (4–6 mm) and grown in Ham’s growth medium (DMEM:F-12 [50:50], 10% FBS plus l-glutamine, antibiotic/antimycotic) as previously described (9). Cells between passages 8 and 12 were used. VSMs (75%–85% confluence) were made quiescent by serum starvation (0% FBS) for 3 days or 0.5% FBS for 6 days, with media change on the third day. Quiescent cells were stimulated with forskolin (10 μM) or the cyclic AMP analog 8-pCPT-2′-O-Me-cAMP (100 μM) for indicated time periods. When using pharmacologic inhibitors, microVSM were treated with inhibitors for 30 min before and during exposure to forskolin or 8-pCPT-2′-O-Me-cAMP.

**Gene delivery.** Complete adenovirus transductions for microVSM were optimized as previously described (9). Adenovirus transductions showed no cytotoxicity and did not affect basal α2C-ARs in control studies (9). The expression of Ad-Rap1A-CA and Ad-Rap1A-DN in human microVSM was determined by Western blot analysis using anti-Rap1 antibody, and was 1.21 ± 0.052-fold for Rap1A-CA (n = 5, P < 0.05), and 1.53 ± 0.18-fold for Rap1A-DN (n = 5, P < 0.05), relative to the control adenovirus (Ad-Control). GAPDH was used as control for sample loadings.

Delivered of plasmid DNA to human microVSM was achieved by electroporation (Amaxa nucleofection, Amaxa, Gaithersburg, MD) of 400,000 cells with 4 μg of total DNA using program U-25 for human smooth muscle cells as previously described (12). When GFP was cotransfected, 3.5 μg of pcDNA3 or Rap1-CA was used with 0.5 μg of GFP (pmaxGFP, Amaxa Biosystems). For confocal imaging studies, electroporated microVSM were plated on glass coverslips. Cells were allowed to recover overnight in 10% Ham’s growth medium. Transfected cells were subsequently washed once with serum-depleted medium (0.5% Ham’s), and quiesced in 0.5% Ham’s for 48–72 h. When inhibitors (fasudil or H-1152) were used, they were added at the time of quiescence with 0.5% Ham’s medium. Cells cotransfected with, and expressing GFP, were identified and used for imaging.

**Rho activity inhibition by C3 exoenzyme.** Quiescent microVSM were pretreated with C3 transerase (0.5 μg/ml) for 2, 4, or 6 h in serum-free medium, followed by stimulation with forskolin (10 μM) or DMSO (control) for 16 h. Cells were fixed and examined for stress fiber formation using confocal microscopy.

**Immunofluorescence and confocal microscopy.** Cells were fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, washed with phosphate-buffered saline (PBS) and blocked with 2% normal goat serum. The cells were incubated with primary antibody for 1 h at room temperature, followed by PBS washes, and incubation with Alexa Fluor-labeled secondary antibody (Molecular Probes, 1 h, room temperature). This was followed by Hoechst nuclear staining (2 μg/ml, 15 min at room temperature), and mounting with anti-fade
mounting medium. Cells were visualized by laser scanning confocal microscopy (×63 water objective, 512 × 512 pixels, LSM510, Zeiss, Germany). Optical slices allowed spatial visualization of α2C-ARs (intracellular versus cell surface, referred to as cell boundary). Secondary Alexa Fluor antibodies used were red fluorescent Alexa Fluor 568 or green fluorescent Alexa Fluor 488 (anti-mouse or anti-rabbit IgG antibodies, H+L conjugate). Fluorescently labeled phalloidin (Molecular Probes, Alexa Fluor 488 phalloidin) was used to detect relative F-actin levels in unstimulated versus stimulated cells. Nuclei were visualized with Hoechst stain. When comparing fluorescence, the highest intensity was used as reference and all images were captured and processed with identical settings and conditions. All the images were processed and presented in their original state, without editing or modifications. Changes in mean fluorescence intensity were quantitated by using the region-of-interest tool, NIS-Elements AR Laboratory Image Analysis System (Nikon Instruments, Melville, NY).

**Rho activation assays.** Human microVSM were grown to 80% confluence and then quiesced in 0.5% FBS-Ham’s. VSM were treated with forskolin (10 μM) or 8-pCPT-2′-O-Me-cAMP (100 μM) for indicated time points. RhoA activation was assessed by precipitation assay for active GTP-bound RhoA using Rhotekin Rho binding domain, according to the manufacturer’s protocol (RhoA pull-down, Upstate Biotechnology, Lake Placid, NY). Rho was detected by Western blotting with anti-RhoA antibody. Alternatively, RhoA activation was performed using RhoA-G-LISA according to manufacturer recommended protocols (Cytoskeleton). Data were normalized to the total RhoA content of each sample and expressed as fold-change in RhoA activity (means ± SE).

**Murine VSM Culture**

Explanted murine VSM were derived from the middle to the distal region of tail arteries of C57BL/6J mice deficient in the Ras-related GTPase Rap1A (murine VSM-Rap1A-null) or control wild-type mice (murine VSM-WT).

The Rap1A<sup>−/−</sup> mice were generated by targeting exon 4 and surrounding sequence and replacing with the neomycin (neo) resistance gene, leading to loss of Rap1A protein expression as previously described (33). Genotyping of tail snips was performed to identify homozygous (+/+) or heterozygous (+/−) for the studies. Tail snips (~2–3 mm) were placed in 200 μl of 50 mM sodium hydroxide and incubated at 95–98°C for 60 min. The reaction was neutralized with 20 μl of 1 M Tris-HCl, pH 8.0. The sample was cleared by centrifugation at 3,000 rpm (956 g) for 5 min. The insertion of neo cassette allows PCR amplification of an 1,107 bp amplicon using primers specific for Rap1A sequence in exon 3 (5′-CTTGCTCTCTGTTACCTATAGGTGCC-3′) and sequence in neo cassette (5′-TCTATCGCCTTCTTATG-3′) (33). The control WT mice showed a PCR amplicon of 1,085 bp using primers specific for Rap1A sequence in exon 3 and exon 4 (5′-CGTGAAAAATCTGTTCTCAGATCTGTC-3′). The mice used for the studies included two male knockouts (Rap1A<sup>Δ−/−</sup>) 7.5 mo and 16 mo of age and a control WT male for comparison (7.5–8.5 mo of age). Explanted cells were grown in Ham’s growth medium. Experiments were performed on cells at passage 7–11 with similar results. The murine VSM from both knockout donors showed similar results for α2C-AR expression, and rescue of α2C-ARs and phosphorylation of serine<sup>113</sup> of filamin-2 (used as control for comparisons) upon introduction of constitutively active Rap1A. All procedures involving live animals were approved by the Ohio State University and the Research Institute at Nationwide Children’s Hospital Institutional Animal Care and Use Committees and conformed to the guidelines of humane and responsible care and use of animals, as mandated by federal regulatory agencies.

The expression of Ad-Rap1A-CA in murine microVSM was determined by Western blot analysis using anti-Rap1 antibody, and was 4.3 ± 1.0-fold (<i>n</i> = 4, <i>P</i> < 0.05), relative to Ad-Control. β-Actin was used as control for sample loadings.

**Western Blot Analysis**

Total cellular protein or whole cell lysates were prepared in lysis buffer (2% SDS/60 mM Tris·HCl, pH 6.8), sonicated and clarified by centrifugation to remove cellular debris. Protein estimations were performed on 20 μl of lysates using the bicinchoninic acid method. Samples (10–40 μg) in 1× loading dye (62.5 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol) were separated on 10% SDS-PAGE for α2C-AR. Samples were incubated at 37°C for 2 h (for α2C-ARs) before loading. The blots were performed as described previously (9). Similar results were seen for α2C-ARs using membrane fractions or whole cell lysates. The results were normalized to β-actin, which was used as control for sample loadings. Alternatively, GAPDH was used as a control for sample loadings. The images were scanned for quantitation using ImageQuant TL software (GE Healthcare).

**HEK293 Studies**

**Cell culture and transient transfections.** Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were routinely maintained in minimal essential medium supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, and without antibiotics. For transient cotransfections, cells were seeded in 100-mm dishes or 12-well plates the day before transfection. Cotransfections in 100-mm dishes were performed with plasmid DNA encoding amino terminus HA-tagged α2C-AR (6.5 μg) plus pcDNA3 (6 μg), constitutively active Rap1A (Rap1A-CA, 6 μg; 2.04 ± 0.67-fold expression compared with the empty control vector pcDNA3, <i>n</i> = 4, determined by Western blot analysis; β-actin was used as control for sample loadings), or ROCK-CA (0, 0.2, 0.5, and 1.0 μg; see Fig. 8E for expression of 0.2 μg). On the day of transfection, medium was changed and DNA was mixed with the FuGene6 reagent (Roche Biochemicals) at a ratio of 3 to 1 [μl FuGene6 to μg nucleic acid], for 20 min. The FuGene and DNA mix was added to the cells.

**Live cell labeling studies.** These studies utilized amino-terminus HA-tagged α2C-ARs for transient transfections. This HA tag is on the extracellular domain of cell surface receptors and allows quantitation of surface receptors by immunoprecipitation and Western blot analysis.

Forty-eight hours after transfection, cells were washed with iced-cold PBS and incubated with HA antibody (1:200, mAb.H11, Covance) for 1 h at 4°C, followed by incubation in lysis buffer (1% Digitonin, 0.5% sodium deoxycholic acid, and protease inhibitors), for 1 h at 4°C. Lysates were centrifuged at 14,000 g for 30 min at 4°C, and equal protein amounts of supernatant were used for antibody-bound receptor immunoprecipitation using protein A-Sepharose 4B beads (1 h at 4°C). Sepharose beads were washed three times with washing buffer (0.1% Digitonin in PBS), incubated in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT) for 1 h at 37°C. The immunoprecipitated receptor was visualized by Western blotting and chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ). When using fasudil, cells were treated with the inhibitor at time of transfection. In all experiments, cell surface expression of receptors was compared with the total cellular expression of the receptor.

**Measurement of intracellular cAMP.** HEK293 cells. Transfected HEK293 cells were pretreated with the phosphodiesterase inhibitor IBMX (0.225 mM; Sigma) for 30 min before exposure to the adenylyl cyclase activator forskolin or the selective α2C-AR agonist UK-14,304 (Sigma). Cells were treated with UK-14,304 (concentration range: 10 PM–10 nM; duplicate treatments with one concentration per well) for 1 min, followed by treatment with forskolin (3 μM) for 5 min. Cells were then placed on ice and washed with ice-cold PBS, lysed with 10% trichloroacetic acid and assayed for intracellular cAMP using an RIA kit (Amersham Biosciences). Results were normalized to control and expressed as fold-change over control, relative to untreated. 

**Upstream Data.** The expression of Ad-Rap1A-CA in murine microVSM was determined by Western blot analysis using anti-Rap1 antibody, and was 4.3 ± 1.0-fold (<i>n</i> = 4, <i>P</i> < 0.05), relative to Ad-Control. β-Actin was used as control for sample loadings.

**Western Blot Analysis**

Total cellular protein or whole cell lysates were prepared in lysis buffer (2% SDS/60 mM Tris·HCl, pH 6.8), sonicated and clarified by centrifugation to remove cellular debris. Protein estimations were performed on 20 μl of lysates using the bicinchoninic acid method. Samples (10–40 μg) in 1× loading dye (62.5 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol) were separated on 10% SDS-PAGE for α2C-AR. Samples were incubated at 37°C for 2 h (for α2C-ARs) before loading. The blots were performed as described previously (9). Similar results were seen for α2C-ARs using membrane fractions or whole cell lysates. The results were normalized to β-actin, which was used as control for sample loadings. Alternatively, GAPDH was used as a control for sample loadings. The images were scanned for quantitation using ImageQuant TL software (GE Healthcare).
Rap1A, Rho-ROCK, and Translocation of Vascular α2C-ARs

In this study we explored the role of Rap signaling in endogenous regulation of α2C-ARs in microVSM. We utilized the novel analog of cAMP, 8-pCPT-2'-O-Me-cAMP, a cell-permeable, potent activator of Epac-Rap1 pathway that has higher affinity for Epac than cAMP (15, 40), to examine the effect on α2C-AR expression in human microVSM. 8-pCPT-2'-O-Me-cAMP (100 μM, 9 h) increased α2C-AR expression, determined by Western blot analysis (3.3 ± 0.8-fold versus control taken as 1; P < 0.05, n = 4). This effect of 8-pCPT-2'-O-Me-cAMP, which activates Epac, was similar to the effect of the adenylyl cyclase activator forskolin on α2C-AR expression (12). Together, these results linked high expression of α2C-ARs in microVSM to cAMP-Rap1 signaling.

RESULTS

Rap1A, α2C-ARs, and Actin Dynamics

We used a non-radioactive ELISA kit (Enzo Life Sciences, Farmingdale, NY). Quiescent microVSM were treated with solvent or the Epac-Rap1-selective activator 8-pCPT-2'-O-Me-cAMP (100 μM) for 14 h, followed by pretreatment with IBMX, and treatments with UK-14,304 (10 pM–10 nM) and forskolin (10 μM), as described above for HEK293 cells.

Statistical Analysis

Statistical analysis of the data was performed by Student’s t-test for either paired or unpaired observations. When more than two means were compared, either a one-way ANOVA with Bonferroni or Dunnett’s post hoc test for multiple comparisons or a two-way ANOVA with Tukey-Kramer’s post hoc test (GraphPad Prism, San Diego, CA) was used to identify differences among groups. Data are presented as means ± SE, where n equals the number of different experiments. Values were considered statistically different when P < 0.05.
effect of Rap1A was seen in human microVSM (Fig. 2B). To confirm the specificity of the effects of Rap1A in microVSM, the expression of Rap1B was examined. Indeed, human and murine (wild-type and Rap1A-null) microVSM showed detectable expression of Rap1B (Fig. 2C).

In control mouse VSMs, α2C-ARs were localized predominantly to a perinuclear compartment in WT and Rap1A-null cells (Fig. 3A). In WT cells, forskolin treatment (10 μM, 12 h) caused translocation of α2C-ARs to trans-cytoplasmic filaments and to the cell surface (Fig. 3, A and B). However, the...
effect of forskolin was abolished in Rap1A-null microVSMs, and α2C-ARs were retained at the perinuclear site as in control cells (Fig. 3, A and B). Similar results were observed with forskolin at 3, 6, and 16 h in Rap1A-null microVSM (not shown). Using the Rap1A-CA rescue approach (in Rap1A-null cells), α2C-ARs translocated to trans-cytoplasmic filaments and to the cell surface (Fig. 3, C and D). Costaining of the cells with fluorescent phalloidin demonstrated that the filaments were F-actin and that Rap1CA caused a dramatic increase in F-actin levels in microVSMs (Fig. 3, C and E). Likewise, in human microVSMs, the Epac-Rap1 activator, 8-pCPT-2′-O-Me-cAMP (100 μM, 9 h), stimulated α2C-AR mobilization.
from perinuclear region to F-actin filaments and to the plasma membrane or cell boundary (Fig. 4A, top, and Fig. 4B). These effects of 8-pCPT-2’-O-Me-cAMP were diminished by adenovirus-mediated transduction of dominant inhibitory Rap1A (Ad-Rap1A-DN, Fig. 4A, bottom, and Fig. 4B). A control adenovirus (Ad-Control) did not show this inhibition (not shown). The role of F-actin in receptor translocation to the cell surface was examined in the presence of the actin destabilizing agent cytochalasin D. Indeed, in 8-pCPT-2’-O-Me-cAMP (100 μM, 9 h)-stimulated microVSM, the α2C-AR translocation from the perinuclear region to F-actin filaments and to the plasma membrane or cell boundary was significantly reduced (Fig. 4, C and D). Similar results were seen with a different actin destabilizing agent, latrunculin A (10 nM, not shown). Cell surface α2C-ARs were Gi-coupled and when activated, they suppress adenyl cyclase production of cAMP (20). Indeed, 8-pCPT-2’-O-Me-cAMP-stimulated microVSM cell surface α2C-ARs were functional and inhibited forskolin-stimulated intracellular cAMP production when activated by the α2-AR-specific agonist UK-14,304 (Fig. 4E).

**Cyclic AMP, Rap1, and RhoA in MicroVSM**

The small GTPase RhoA is a central regulator of actin cytoskeleton reorganization. The Rho effector kinase ROCK mediates key signaling events, leading to increased accumulation and stability of F-actin filaments (21, 27). Our results demonstrated increased F-actin levels and colocalization of α2C-ARs on actin filaments and cell boundary following Epac-Rap1 activation implicating F-actin dynamics in receptor trafficking. To validate these observations, we assessed Rho activation in human microvsm using RhoA-GTP pull-down assays. The Epac-Rap1 activator 8-pCPT-2’-O-Me-cAMP or the adenyl cyclase activator forskolin activated microVSM Rho with different kinetics (4.1 ± 1.3-fold and 3.6 ± 0.8-fold vs. control unstimulated cells, respectively, at 10 min, n = 3, P < 0.01, Fig. 5). This effect of 8-pCPT-2’-O-Me-cAMP (100 μM), however, was not observed in Rap1A-null microVSM (0.86 ± 0.16-fold vs. control unstimulated cells, at 10 min, n = 3, P = not significant). Indeed, the increase in F-actin levels stimulated by forskolin or 8-pCPT-2’-O-Me-cAMP was inhibited by the cell-permeable Rho inhibitor C3 transferase (0.5 μg/ml) or by inhibition of ROCK with fasudil (3 μM) or H-1152 (0.1 μM) (Fig. 5, A–D). Likewise, the increase in F-actin occurring in Rap1A-CA-transfected cells was reduced following ROCK inhibition (fasudil 3 μM or H-1152 0.1 μM) (Fig. 7, A–E).

**Role of Rap1 and Rho in Receptor Translocation to the Cell Surface**

Our studies suggest that the Rap1A signaling pathway may be involved in the mobilization of α2C-ARs to the cell surface. To directly test the role of Rap1A in α2C-AR surface mobilization, cotransfection studies were utilized in α2-AR-deficient HEK293 cells using a live cell labeling approach to quantitate...
surface receptors (Fig. 8A) (1). α2C-ARs are normally retained in the perinuclear trans-Golgi compartment at 37°C in these cells (11, 30). These receptors are Gt-coupled and when cell surface α2C-ARs are activated, they suppress adenylyl cyclase production of cAMP (20). Cell surface localization and receptor coupling and function, therefore, can be quickly and easily measured by using this approach.

Live cell labeling was performed using expression plasmids for constitutively active Rap1A-CA and amino-terminus HA-tagged α2C-ARs. Rap1A-CA significantly increased cell surface α2C-ARs (Fig. 8B, normalized for total cellular levels of the receptor). ROCK inhibition with fasudil (3 µM) attenuated this effect and reduced the localization of α2C-ARs to the cell surface (Fig. 8C). Similarly constitutively active ROCK (ROCK-CA) increased cell surface α2C-ARs (Fig. 8D and E). These receptors were functional and inhibited forskolin-stimulated intracellular cAMP production when activated by the α2-AR-specific agonist UK-14,304 (Fig. 8F).

**DISCUSSION**

The α2-adrenoceptors (α2-ARs), consisting of α2A, α2B, and α2C subtypes, are expressed on contractile VSM cells in the microcirculation, for example in arterioles, but not large arterioles...
ies (9, 18, 20, 41, 43). These arterioles determine blood flow to organs and generate peripheral resistance (43). Unlike plasma membrane-directed α2A- and α2B-ARs (41, 47), α2C-ARs are perinuclear, sequestered in the trans-Golgi network at physiological temperatures, and are not accessible to physiological stimulation and activation (6, 11, 30). In this study we examined the role of the small GTPase Rap1A in α2C-AR expression and localization. Our studies show a unique physiological role of Rap1A in the microcirculation. We demonstrate that in human microVSM, activated Rap1A is coupled to increased expression of α2C-ARs and to increased translocation of perinuclear α2C-ARs to the cell surface. Intriguingly, the Rap1A-induced mobilization of α2C-ARs to the cell surface is mediated by activation of RhoA and therefore appears to be acting in a similar manner to cold-induced mobilization and functional rescue of the receptors.

In human microVSM, increased production of intracellular cAMP (by direct activation of adenylly cyclase by forskolin, or activation of G_{i} by cyclooxygenase-2 derived prostanoids, β-ARs, or cholera-toxin) markedly increases expression of endogenous α2C-ARs (8). Cells treated with forskolin or cholera toxin show activation of the PKA substrate cAMP responsive element-binding protein (CREB), phosphorylating CREB within 10 min of treatment (8). Pharmacological inhibition of A-kinase with H-89 inhibits CREB phosphorylation but shows no detectable effect on α2C-AR expression, suggesting selective and compartmentalized A-kinase signaling within the microVSM. The A-kinase, however, has the potential to suppress α2C-AR expression in microVSM (8). Indeed, in murine Rap1A-null microVSM, an inhibitory effect of forskolin on α2C-AR expression was observed, likely the A-kinase suppressive effect visible physiologically in the absence of Rap1A. These results therefore suggest that in

![Fig. 7. Rap1A-Rho link in microVSM. Human microVSM were transiently cotransfected with empty control vector (pcDNA) and plasmids expressing green fluorescent protein (GFP) (A) or cotransfected with plasmids expressing constitutively activated Rap1A (Rap1A-CA) and GFP (B) and examined for F-actin using fluorescently labeled phallloidin (Alexa Fluor 568 phallloidin). Cells were examined in the absence (A and B) or presence of ROCK inhibitors fasudil (3 μM) (C) or H-1152 (0.1 μM) (D). Nuclei were visualized with Hoechst stain (blue). Scale bars, 20 μm. E: F-actin quantitation. Data from 4–6 cells per condition are shown. *P < 0.05.]
microVSM, cAMP activates both the A-kinase and the Epac-Rap1 pathways, and that cAMP-Epac-Rap1-JNK-c-jun signaling predominates in transcriptionally activating the \( \alpha_{2c} \)-AR gene. Together, these findings suggest that the \( \alpha_{2c} \)-AR expression can be modulated locally in the microcirculation. For example, vascular injury, followed by inflammation, can activate cAMP-Epac-Rap1-JNK-c-jun signaling, potentially leading to increased intracellular pool of \( \alpha_{2c} \)-ARs.
In microVSM, the α2C-ARs mobilized from the perinuclear region to F-actin and the plasma membrane. We saw similar results in murine wild-type microVSM, coupling actin dynamics to receptor translocation. In the absence of Rap1A (Rap1A-null murine microVSM) the actin dynamics were not seen and the perinuclear receptors failed to translocate. This defect could be rescued by delivery of constitutively active (CA) mutant of Rap1A, which not only increased expression of α2C-ARs, but increased translocation. It is possible that increased expression may have caused saturation of the microVSM retention systems and overflow of receptors to the cell surface. However, when we utilized heterologous HEK293 cells, which do not express endogenous α2-ARs, to test the role of Rap1A or Rho effector ROCK in α2C-AR translocation using a live cell labeling approach, we saw that Rap1A-CA or Rho-kinase ROCK-CA specifically caused translocation of functional α2C-ARs to the cell surface. These results suggested that the expression and translocation of α2C-ARs are independent and involve two separate processes.

In VSM phosphorylation of the ROCK substrate, the myosin light chain phosphatase (MLCP) regulatory subunit, known as MYPT1, leads to inhibition of MLCP activity, and increase in myosin light chain (MLC) phosphorylation, force generation, and stress fiber formation (16, 32). Inhibition of ROCK activity, for example, by pharmacological inhibitors or dominant inhibitory Rap1A which sequesters Epac, would therefore contribute to reduced MYPT1 phosphorylation and increased phosphorylase activity, leading to MLC dephosphorylation, disassembly of stress fibers, and the loss of cell shape. In support of this notion, we observed increased GTP-bound activated RhoA and reorganized actin cytoskeleton with increased F-actin in microVSM with forskolin or selective Epac-Rap activation by the cAMP analog 8-pCPT-2’-O-Me-cAMP. However, RhoA was not activated and F-actin was absent in Rap1A-null cells or diminished in cells with Rho or ROCK inhibitors or dominant inhibitory Rap1A, with apparent alteration in cell shape. These results support the role of Rap1A in activating Rho-ROCK signaling in microVSM and are in contrast to observations made in large vessels where increased Rap1 activity contributes to decreased RhoA activity and function (57).

Collectively, our results show that Rap1A regulates α2C-AR transcription (12) and also enhances α2C-ARs translocation to the cell surface. This effect of Rap1 is mediated through the activation of Rho-ROCK signaling and the actin cytoskeleton. Our results suggest regulated cell surface delivery of the α2C-AR subtype by cAMP-Epac-Rap1-Rho-ROCK and are consistent with its role as a “stress-responsive” receptor of the microvasculature (9). The α2C-ARs have a discrete physiological role in the microvasculature and engage with the cell surface under select conditions to elicit a vasoconstrictor response (6). Our results also show that Rap subtypes, specifically Rap1A, have a discrete function in microVSM. For example, no compensation by other Rap subtypes was apparent in murine Rap1A-null microVSM. In these cells, the α2C-AR inducibility by cAMP was dramatically affected. Our studies suggest that despite the species differences, α2C-AR regulation is remarkably similar in human and mouse.

Vascular α2C-ARs were originally considered to be vestigial or silent receptors. However, in cutaneous arteries, moderate cooling to 28°C increases functional activity of α2C-ARs, leading to augmented vasoconstriction (1, 2, 7, 30, 48). This effect of cooling on α2C-AR function is mediated by RhoA-ROCK signaling. Cooling triggers a rapid increase in mitochondrial reactive oxygen species in VSM, leading to RhoA-ROCK activation and translocation of α2C-ARs to the cell surface, enabling enhanced α2-AR reactivity and cold-induced vasoconstriction (1, 2). In the cutaneous circulation, this augmented constriction at 28°C physiologically has a protective role, reducing body heat loss and directing blood to the major organs to maintain the body core temperature (49). This augmented receptor biological activity is reversible, and is lost when temperature returns to 37°C (7). In support of these findings, recent in vivo studies have implicated the role of cooling-triggered Rho-ROCK-α2C-AR-mediated vasoconstriction in mouse and human skin (25, 48).

The present study shows for the first time that functional rescue of α2C-ARs is not restricted to cold exposure but can also occur at normal physiological temperatures of 37°C. This may have profound implications for human conditions in which α2C-ARs have been implicated, for example, during the progression of peripheral vascular disorder such as Raynaud’s phenomenon (3, 17). The signaling pathway identified in this study may contribute to severity of vaso spasstic episodes. The cAMP-Rap1 signaling pathway can increase intracellular pool of α2C-ARs. Consequently, increased expression and translocation of functional α2C-ARs by convergence of parallel Rap1-Rho-ROCK and cooling-Rho-ROCK pathways may contribute to augmented cell surface mobilization of α2C-ARs and to pathophysiological responses in the vascular system, particularly to disease progression and disease severity. Alternatively, it is possible that cooling may also activate Rap1 signaling contributing to receptor expression and translocation, and that signaling in series may occur. Indeed, the Rap1A-

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**Fig. 8.** Rap1-Rho-ROCK signaling and α2C-AR translocation to the cell surface. A: quantitation of cell surface α2C-ARs in HEK293 cells performed by live cell labeling and immunoprecipitation of the receptor’s extracellular amino-terminus hemagglutinin (HA) tag. B: the effect of constitutively active Rap1A (Rap1A-CA) on cell surface localization of α2C-ARs in HEK293 cells. Data were normalized to the total cellular expression of α2C-ARs (not shown). The results are expressed as fold change in surface expression from control values and are presented as means ± SE (n = 8, *P < 0.01). Rap1A-CA increased surface expression of α2C-ARs by 3.93 ± 0.75-fold. C: the effect of the ROCK inhibitor fasudil (3 μM) on Rap1A-CA-mediated α2C-AR mobilization in HEK293 cells. Cell surface expression was assessed by live cell labeling and immunoprecipitation of α2C-ARs. Data were normalized to the total α2C-ARs expression (not shown). Results are expressed as fold change in surface expression from control values and presented as means ± SE (n = 3, *P < 0.05 significantly different from control; **P < 0.01. D: the effect of constitutively active Rac kinase (ROCK-CA) was examined using increasing concentrations of ROCK-CA expressing plasmid, 0, 0.2, 0.5, and 1.0 μg, on cell surface localization of α2C-ARs in HEK293 cells, assessed by live cell labeling and immunoprecipitation of α2C-ARs. Data were normalized to total cellular expression. Results are expressed as fold change in surface expression vs. control values and presented as means ± SE (n = 3, *P < 0.05). E: expression of myc-tagged p60-ROCK3, carboxyl-terminus truncated HEK293 cells (lane 1) or in cells cotransfected with α2C-ARs and ROCK-CA (2 μg, lane 2). F: the effect of ROCK-CA on α2C-AR function in HEK293 cells. Receptor function was assessed by measuring intracellular cyclic AMP accumulation in response to the α2-AR agonist UK-14,304 (UK; 10^-8 M to 10^-11 M). Forskolin (FSK) was used to elevate the basal (B) level of cyclic AMP. Data are presented as a percentage of the response to forskolin alone and are expressed as means ± SE; n = 3, **P < 0.01.
null murine microVSM model identified in this study will be a valuable tool to investigate these possibilities.

In other non-VSM cells, Rap1 activation and on-off cycling has been linked to alteration in cytoskeletal dynamics during migration, formation of actin-rich pseudopodial protrusions for extravasation, and metastasis of cancer cells (19), and chemokine-induced cell migration (36). However, in sharp contrast to the positive association between Rap1 and actin dynamics identified in these cell-types, activated Rap1 decreases thrombin-induced RhoA activity and Rho-induced stress fibers in endothelial cells, where Rap1 regulates endothelial barrier function and integrity by enhancing cortical actin (10, 42). Similarly, in the rat phaeochromocytoma cell line PC12, nerve growth factor (NGF) treatment stimulates activation of Rap1 and inactivation of Rho, necessary for neuronal differentiation (24, 28, 29, 38, 46, 53, 55). An intriguing observation in treated PC12 cells is that NGF causes concentration and colocalization of α2C-ARs in neurite outgrowths rich in F-actin (26). It is possible that, in neuronal cells, α2C-AR translocation to the cell surface is mediated by a Rap1-dependent, RhoA-independent mechanism or is entirely independent of Rap1-signaling. Altogether, these observations point to differences in Rap1 effector coupling that activate (through Rho-GEF) or inhibit Rho (through RhoGAP) in a cell-type-dependent manner.

In summary, our results show Rap1 coupling to RhoA activation and reorganization of the actin cytoskeleton, necessary for translocation of microVSM α2C-ARs. The exact mechanism for α2C-AR translocation by actin remains to be determined and warrants further study. Overall, our results also suggest that Rap1 may have a broader role in microVSM biology. For example, VSM actin dynamics plays a vital role in modulating signaling and gene regulation. Rho-ROCK activity is known to modulate VSM differentiation-specific transcription by modulation of actin-treadmilling (the G to F actin ratios), which in turn can modulate serum-response factor (SRF) cytoplasmic-nuclear localization (34, 35). This modulation of SRF activity modulates SRF-dependent transcription of VSM-specific genes. Furthermore, myocardin-related transcription factors (MRTFs) are actin regulated transcriptional coactivators, which bind G-actin. In response to signal-induced actin polymerization such as Rho activation and concomitant G-actin depletion, MRTFs accumulate in the nucleus and activate target gene transcription (5, 37, 45). Similarly, the actin cytoskeleton is known to have a role in protein trafficking (13, 51). Rap1 may therefore modulate microVSM differentiation-specific gene expression by modulating actin dynamics, and similar to α2C-ARs, regulate trafficking of otherGPCRs. The human and murine microVSM utilized in this study will serve as valuable models to examine this provocative role of Rap1.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.C.J. and M.A.C. conception and design of the research; S.C.J., N.T.U., A.H.E., S.M., N.P.E.-D., and M.A.C. performed the experiments; S.C.J., N.A.F., and M.A.C. analyzed the data; S.C.J. and M.A.C. interpreted the results of the experiments; S.C.J., N.A.F., and M.A.C. prepared the figures; S.C.J. and M.A.C. drafted the manuscript; L.A.Q. contributed reagents; M.A.C. approved the final version of the manuscript; S.C.J., N.A.F. and M.A.C. edited and revised the manuscript.

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