Molecular Determinants of the Human α_{2C} -Adrenergic Receptor Temperature-Sensitive Intracellular Traffic[§]

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ABSTRACT

The human α_{2C} -adrenergic receptor (α_{2C} -AR) is localized intracellularly at physiologic temperature. Decreasing the environmental temperature strongly stimulates the receptor transport to the cell surface. In contrast, rat and mouse α_{2C} -AR plasma membrane levels are less sensitive to decrease in temperature, whereas the opossum α_{2C} -AR cell surface levels are not changed in these conditions. Structural analysis demonstrated that human α_{2C} -AR has a high number of arginine residues in the third intracellular loop and in the C-terminus, organized as putative RXR motifs. Although these motifs do not affect the receptor subcellular localization at 37°C, deletion of the arginine clusters significantly enhanced receptor plasma membrane levels at reduced temperature. We found that this exaggerated transport of the human receptor is mediated by two functional arginine clusters, one in the third

intracellular loop and one in the C-terminus. This effect is mediated by interactions with COPI vesicles, but not by 14-3-3 proteins. In rat α_{2C} -AR, the arginine cluster from the third intracellular loop is shifted to the left due to three missing residues. Reinsertion of these residues in the rat α_{2C} -AR restored the same temperature sensitivity as in the human receptor. Proteomic and coimmunoprecipitation experiments identified pontin as a molecule having stronger interactions with human α_{2C} -AR compared with rat α_{2C} -AR. Inhibition of pontin activity enhanced human receptor plasma membrane levels and signaling at 37°C. Our results demonstrate that human α_{2C} -AR has a unique temperature-sensitive traffic pattern within the G protein-coupled receptor class due to interactions with different molecular chaperones, mediated in part by strict spatial localization of specific arginine residues.

Introduction

Thermoregulation is a highly regulated process in homeothermic mammals, which is required to maintain the body temperature independent of the ambient temperature (Morrison 2011; Romanovsky, 2014). Cutaneous circulation plays a major role in this process, functioning as a heat insulator below the epidermal surface (Johnson and Kellogg, 2010; Morrison 2011). Exposure to cold in homeothermic mammals leads to local vasoconstriction, a physiologic response limiting heat loss (Flavahan, 2008). As in all vascular beds, cutaneous circulation is controlled by the autonomic nervous system and local endogenous mediators (Morrison, 2011). However, in certain individuals the local vasoconstriction in response to cold is exaggerated and accompanied by triphasic color changes of

the affected skin region, i.e., pallor, cyanosis, and red flushing (Pope, 2007; Prete et al., 2014). These features define Raynaud's phenomenon, a disease described first by Maurice Raynaud in 1862 (Raynaud, 1862). Raynaud's phenomenon is regarded as a rare disease; however, it is in fact commonly encountered clinically and affects between 3 and 10% of the general population, with a higher incidence in women (Brand et al., 1997; Prete et al., 2014). Although Raynaud's phenomenon was described 150 years ago, a specific treatment is still missing today. Therapy consists of palliative measures associated with non-specific smooth muscle relaxants (calcium channel blockers and nitrates), and no specific biomarkers for early identification of individuals susceptible to Raynaud's phenomenon have been identified.

Several mechanisms have been proposed to mediate the exaggerated vasoconstriction in Raynaud's phenomenon, such as enhanced endothelin levels, augmented serotonin release, or increased activation of the renin-angiotensin system; however, experimental findings do not support the involvement of these factors (Pope et al., 2000; Wood and Ernst, 2006; Nguyen et al., 2010). In contrast, the role of α_2 -adrenergic receptors (α_2 -ARs) in cold-induced vasoconstriction was demonstrated in the mid-1990s by results showing that vasospastic attacks in Raynaud's

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ABBREVIATIONS: AR, adrenergic receptor; cAMP, cyclic adenosine monophosphate; GPCR, G protein-coupled receptor; MS, mass spectrometry; OK, opossum kidney; RX821002, 2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1H-imidazole; UK14304, 5-bromo-N-(2-imidazolin-2-yl)-6-quinoxalinamine; VSMC, vascular smooth muscle cell.

phenomenon patients were prevented by the α_2 -AR antagonist yohimbine, but not by the α_1 -AR blocker prazosin (Freedman et al., 1995). These studies in vivo were complemented by findings in isolated human skin arterioles and mouse or rat tail arteries, showing that the contractile response to moderate cooling is reduced by α_{2C} -AR antagonists (Chotani et al., 2000; Bailey et al., 2004; Filipeanu et al., 2011a). Evaluation of the subcellular localization of α_{2C} -AR in vascular smooth muscle cells (VSMCs) and fibroblasts confirmed that at 37°C this receptor accumulates in the endoplasmic reticulum (Daunt et al., 1997; Angelotti et al., 2010; Filipeanu et al., 2011a). Interestingly, in cells with a neuronal phenotype, α_{2C} -AR displayed strong plasma membrane localization, indicating that its intracellular trafficking is cell dependent (Hurt et al., 2000). We have demonstrated that intracellular accumulation of α_{2C} -AR at 37°C is not related to aberrant receptor internalization/recycling but to defects in export trafficking, which can be corrected by nonspecific pharmacological chaperones such as dimethylsulfoxide or glycerol (Filipeanu et al., 2011a). We have also identified HSP90 as an interacting partner of α_{2C} -AR and have shown that inhibition of HSP90 activity increases the α_{2C} -AR traffic to the cell surface at 37°C but has no effect at 30°C. Furthermore, we found that in VSMCs from rat tail artery, exposure to 30°C decreased HSP90 (but not HSP70) cellular levels (Filipeanu et al., 2011a). These results indicate that fine interactions with specific molecular chaperones can be responsible for temperature-dependent α_{2C} -AR traffic.

α_{2C} -AR belongs to the seven G protein-coupled receptor (GPCR) superfamily, the largest family of the human genome and the target for about 30–40% of current therapeutic drugs (Duvernay et al., 2005; Dong et al., 2007). In this class, certain GPCRs were shown to be exported in an inefficient manner and only a fraction of the synthesized receptors reach the plasma membrane, such as the δ -opioid receptors and the human gonadotropin releasing hormone receptor (Petaja-Repo et al., 2000; Re et al., 2010). The transport of the GPCR to the plasma membrane critically depends on the interactions at different steps of various accessory proteins, which are also called molecular chaperones (Dong et al., 2007). These accessory proteins are heterogeneous and can interact in a highly specific manner with individual GPCRs, such as AHA1 (a HSP90 cochaperone) for CB1R (Filipeanu et al., 2011b). Also, the cellular levels of these accessory proteins can be cell-type specific and could be altered in the diseased state, and therefore may change the expression, subcellular localization, and functional responses of different GPCRs. Based on this concept, in the present work we further focused on the elucidation of the mechanisms modulating α_{2C} -AR intracellular traffic and on identifying interacting partners that modulate the receptor temperature-dependent traffic.

Materials and Methods

Cell Culture and Transfection. HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA) and the experiments were performed in passages 7–20. The cells were cultured in Dulbecco's modified Eagle's medium and supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (all cell culture reagents were obtained from Life Technologies, Carlsbad, CA). Opossum kidney (OK) cells (cortex proximal tubule epithelial cells) were a gift from Dr. Guangyu Wu (Medical College of Georgia, Augusta, GA) and were grown in similar conditions as the HEK293T cells. Human coronary VSMCs were a gift

from Dr. T. Cooper Woods (Tulane University, New Orleans, LA) and were routinely cultured in full SmGM-2 Growth Medium (Lonza, Walkersville, MD). Transient transfection of the HEK293T cells (70–80% confluent) was carried out in 6-well plates using LipofectAMINE 2000 (Life Technologies) for 6 hours with a cDNA:liposome ratio of 2.5:1 as previously described (Filipeanu et al., 2011a; Deshotels et al., 2014). Six hours later the cells were trypsinized and plated at a density of 10^6 cells/well in 6-well plates for Western blot experiments, or 4×10^5 cells/well in 12-well plates for radioligand binding experiments and cyclic adenosine monophosphate (cAMP) determination. Based on examination under a fluorescent microscope, about 85–90% of the cells were transfected under these conditions. Human coronary VSMCs were transfected using the Neon Electroporation System (Life Technologies) with two pulses of 1475 V and 20 millisecond duration. The transfection efficacy under these conditions was found to be about 50%. The experiments were performed 72 hours after electroporation with the cells being kept in the SmGM-2 medium without growth supplements for the last 6 hours.

Protein Interaction with α_{2C} -AR Identification by Liquid Chromatography–Mass Spectrometry Analysis. The experiments were carried as previously described (Filipeanu et al., 2011b). Cell lysates from human and rat α_{2C} -AR-transfected HEK293T cells were incubated overnight with 20 μ l/mg anti-GFP Agarose (MBL International, Woburn, MA), and subsequently harvested into 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 20% glycerol (Sigma-Aldrich, St. Louis, MO). The resulting mixture was subjected to a brief centrifugal step before determining the protein concentration using the Bradford method (Bio-Rad, Hercules, CA). Two-dimensional difference gel electrophoresis labeling and analysis was performed as previously described (Filipeanu et al., 2011b). Fluorophore-labeled protein gels were scanned using a Typhoon 9400 Variable Mode Imager (GE Healthcare, Piscataway, NJ) at 100 μ m resolution. CyDye (GE Healthcare) difference gel electrophoresis labeling was detected using the following wavelength settings: Cy2, excitation 488 nm, emission 520 nm; Cy3, excitation 532 nm, emission 580 nm; and Cy5, excitation 633 nm, emission 670 nm. Spot detection and quantification were performed using the DeCyder differential analysis software DIA, version 5.0 (GE Healthcare) and individual gels were imported into DeCyder biologic variation analysis for *t* test analysis. Spots of interest were determined to be any difference greater than 50% or a *t* test *P* value of less than 0.05. Gels were then post stained with Sypro Ruby (Bio-Rad), and images were captured again using the Typhoon 9400 Imager (GE Healthcare). Spots of interest were excised and trypsin (Promega, Madison, WI) digested using the automated Spot Handling Workstation (GE Healthcare).

The mass spectrometry (MS) data were acquired using a Thermo LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to an Eksigent nanoLC (Dublin, CA). Peptide samples were loaded onto a Dionex C18 PepMap 100 trap column with dimensions of 300 μ m (i.d.) $\times$ 5 mm (Dionex, Sunnyvale, CA) and were separated by a reversed-phase C18 PicoFrit emitter with dimensions of 75 μ m (i.d.) $\times$ 10 cm (bed length) with 15 μ m tip size (New Objective, Woburn, MA). Peptides were loaded at 500 nl/min using a mobile phase of 2% acetonitrile and 0.1% formic acid and then eluted using a gradient of 5–40% acetonitrile and 0.1% formic acid over 16 minutes, with a ramp to 60% acetonitrile and 0.1% formic acid for 10 minutes, and finally a ramp to 95% acetonitrile and 0.1% formic acid for 10 minutes. A top five data-dependent scan strategy was used. The MS1 scan range was between *m/z* 300 and 2000. The top five most abundant peptides in this MS1 scan were chosen for MS/MS. The MS/MS parameters were the following: isolation window set to 2 Da, 35% relative collision energy, dynamic exclusion enabled with repeat count set to 1, repeat duration of 30 seconds, and an exclusion size of 100 with an exclusion duration of 20 seconds.

The MS data were then analyzed by Proteome Discoverer 1.4 (Thermo Fisher Scientific) against the Human SwissProt database (<http://www.uniprot.org/proteomes/>). Database searching was performed

for b and y ion series and up to 1 missed trypsin cleavage. The precursor mass tolerance was set to 1.2 Da and the fragment mass tolerance set to 0.8 Da. Methionine oxidation and cysteine carbamidomethylation were considered as possible modifications.

Antibodies and Chemicals. The antibodies used in the present study were obtained from the following sources: anti- α_2C -AR antibody from Abcam (Cambridge, MA); anti-GFP Agarose from MBL International; anti-GFP, anti-14-3-3 ζ , anti- β -COP, anti-pontin, anti- β -actin, anti-phospho-ERK 1/2, and anti-total ERK 1/2 from Santa Cruz Biotechnology (Dallas, TX); and anti-mouse and anti-rabbit horseradish peroxidase–secondary antibodies from Perkin-Elmer (Waltham, MA). Pontin small hairpin RNA lentiviral particles and puromycin were obtained from Santa Cruz Biotechnology, whereas β -COP small hairpin RNA was obtained from Life Technologies. Rottlerin was obtained from Sigma-Aldrich and forskolin and UK14304 [5-bromo-*N*-(2-imidazolin-2-yl)-6-quinoxalinamine] were obtained from Tocris (Minneapolis, MN).

Plasmid Constructions and Generation of the α_2C -AR Mutants. GFP-tagged human α_2C -AR and α_2B -AR at their C-termini were generated by polymerase chain reaction after the stop codon was mutated, and the sequences restricted with HindIII/SalI in frame with GFP were ligated into the pEGFP-N1 vector (Clontech, Mountain View, CA), as previously described (Filipeanu et al., 2011a). Flag-tagged rat α_2C -AR, a kind gift from Dr. Randy Hall (Emory University, Atlanta, GA), was used to generate GFP-tagged rat α_2C -AR by a similar procedure. As demonstrated previously (Filipeanu et al., 2011a), these GFP-tagged constructs have similar binding properties as nontagged receptors.

All receptor mutants were obtained using the Quick Change site-directed mutagenesis kit (Agilent, Santa Clara, CA). The following primers were used to obtain the following mutants (where the mutation is shown in bold italic characters):

Human α_2C -AR²⁴⁰ATR²⁴²: 5' gtg gcc aag ctg **cg**c acg cgc acg ctc 3'; 3' gtg gcc aag ctg **ggc** acg cgc acg ctc 5';
 Human α_2C -AR²⁹⁴RRAAR²⁹⁸: 5' gca cgc gcc gag agg cgg **cg**g **cg**c cgg ggc cgc ttg cgg cgg 3'; 3' gca cgc gcc gag agg cgg **gct** **gct** cgg ggc cgc ttg cgg cgg 5';
 Human α_2C -AR³⁰⁶RRA³⁰⁸: 5' cgg ggc ggg cgg cgg **gca** cgc ggc cgc gag ggg 3'; 3' cgg ggc ggg cgg cgg **gca** cgc ggc cgc gag ggg 5';
 Human α_2C -AR³⁵⁹RRAAR³⁶⁴: 5'tc ttc ctg tgc cgc cgg **cg**c **cg**g cgc cgc agc agc gtg tg3'; 3'tc ttc ctg tgc cgc cgg **gcc** **gcg** cgc cgc agc agc gtg tg5';
 Human α_2C -AR⁴⁵⁸RRAAR⁴⁶²: 5'atc ctc ttc cga cgg **agg** **aga** agg ggc ttc agg cag3'; 3'atc ctc ttc cga cgg **gcg** **gca** agg ggc ttc agg cag5';
 Rat α_2C -AR²⁶⁹GA²⁷⁰: 5' gtt ggg caa ggc ggc **ggg** **ggc** cgg aga gaa tgg cgc gcc 3'; 3' ggc cgc caa tgc cca **tct** **tct** cgc gcc ccc gcc gcc tgg ccc aac 5';
 Rat α_2C -AR 290A: 5' gtg gag ccg gat gag **agc** **agc** gcc cgc gca gag agg cgg agg cg 3'; 3' cgc ctc cgc ctc tct gcc cgc **ctg** **ctc** tca tcc gcc tcc ac 5'; and
 Rat α_2C -AR³²⁶G: 5' cgg acc ggg acc tgg gtt **ggc** agc ggc cga aca ggg tgc tgc 3'; 3' cga gca ccc tgt tgc **ggc** gct gcc aac cca ggt ccc ggt ccg 5'.

The α_2C -AR/ α_2B -AR chimeric construct was generated by inserting the selected sequence using BamHI restriction sites introduced by polymerase chain reaction. The sequence of each construct was confirmed by restriction mapping and nucleotide sequence analysis (Integrated DNA Technologies, Coralville, IA).

Coimmunoprecipitation. Immunoprecipitation of the receptors was performed in a similar manner as previously described (Filipeanu et al., 2011a; Deshotels et al., 2014). In brief, HEK293T cells were cultured on 10 cm² dishes and transfected at ~80% confluency with 10 μ g of GFP-tagged human or rat α_2C -AR for 48 hours, fetal bovine serum being omitted during the last 18 hours. The cells were washed twice with phosphate-buffered saline and lysed in 300 μ l of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and Complete Mini protease

inhibitor cocktail (Roche, Indianapolis, IN). After gentle rotation for 1 hour, the samples were centrifuged for 15 minutes at 14,000g and the supernatant was incubated with 50 μ l of Protein G Sepharose (Santa Cruz Biotechnology) for 1 hour at 4°C to remove nonspecific bound proteins. The samples were then incubated with 5 μ g of anti-GFP antibodies overnight at 4°C with gentle rotation followed by incubation with 50 μ l of Protein G Sepharose beads for 5 hours. Resin was collected by centrifugation and washed four times with 500 μ l of lysis buffer. Immunoprecipitated receptors were eluted with 100 μ l of 1 $\times$ SDS-PAGE loading buffer, separated by 10% SDS-PAGE and visualized by immunoblotting using specific antibodies.

Western Blotting. The cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The signal was detected using ECL Plus (Bio-Rad) and a Fuji Film luminescent image analyzer (LAS-1000 Plus) and quantitated using the Image Gauge program, version 3.4 (GE Healthcare).

Radioligand Binding. The experiments were carried out as previously described (Filipeanu et al., 2006, 2011a) on intact cells plated on 12-well plates at a density of 5×10^4 cells/well. The cells were serum starved after 24 hours to prevent differential proliferation at different temperatures. Some cells were incubated at 30°C for 18 hours prior to measurements. The determinations were performed at 4°C in 400 μ l of Dulbecco's modified Eagle's medium containing 20 nM [³H]RX821002 [2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1H-imidazole]. Then, the medium was aspired and the cells were washed three times with Dulbecco's modified Eagle's medium, digested with 1 M NaOH, and then the bound radioactivity was determined in a β -scintillation counter. The nonspecific binding determined in parallel experiments in the presence of nonradioactive rauwolfscine (10 μ M) represented less than 10% of the total radioactivity and was subtracted from the presented results. In these experimental conditions, the internalization of the ligand is minimal (~5%) as was shown by our previous experiments (Filipeanu et al., 2011a).

cAMP Determinations. cAMP cellular levels were determined using a cAMP enzymeimmunoassay system (Cayman Chemical Company, Ann Arbor, MI) as previously described (Filipeanu et al., 2011a,b). The HEK293T cells on 10 cm² plates at ~80% confluency were transfected with 3 μ g α_2C -AR receptor constructs. After 6 hours the cells were plated on 12-well plates at a density of 5×10^4 cells/well. Subsequently, the cells were serum starved for 24 hours and then incubated at 37°C or at 30°C for 18 hours. One hour before stimulation the medium was changed to phosphate-buffered saline supplemented with isobutylmethylxanthine (100 μ M). Then, the cells were incubated with 10^{-8} M UK14304 for 5 minutes, followed by stimulation with forskolin (3 μ M) for 15 minutes. The reactions were stopped by aspirating the medium and addition of 200 μ l of acetic acid (4%). Twenty microliters of cell lysate was then transferred into a microtitre plate and the cAMP levels were determined by enzyme-linked immunosorbent assay according to the manufacturer's protocol.

Statistical Analysis. The experiments were repeated at least in three different cell passages. The statistical differences were evaluated using Student's *t* test, with *P* < 0.05 being considered statistically significant. Data are expressed as the mean $\pm$ S.D.

Results

Previous studies have demonstrated that α_2C -AR accumulates in the endoplasmic reticulum at 37°C and displays little localization at the plasma membrane, which is the common functional site of GPCRs (Daunt et al., 1997; Angelotti et al., 2010; Filipeanu et al., 2011a). Reducing the environmental temperature to 28–30°C greatly enhanced the plasma membrane receptor levels. This particular receptor localization pattern was observed for both heterogeneously human α_2C -AR-transfected cell lines and in VSMCs from rodent tail artery or human cutaneous arteries, which endogenously express this receptor subtype (Chotani et al., 2000; Bailey et al., 2004;

Filipeanu et al., 2011a; Jeyaraj et al., 2012). However, during our previous investigation (Filipeanu et al., 2011a) we observed that the effects of low temperature are higher on the translocation of human α_{2C} -AR-transfected HEK293T compared with endogenous rat α_{2C} -AR in VSMCs from rat tail artery. We analyzed in detail the plasma membrane receptor levels in human α_{2C} -AR-transfected HEK293T cells, in mouse and rat VSMCs, and in the OK cell line by radioligand binding. The OK cell line was included because α_{2C} -AR is expressed endogenously and it has been the historically typical choice to study this receptor subtype before heterogenous transfection was available (Bylund et al., 1992). Incubation at 30°C was performed for 18 hours, the time previously demonstrated by us to induce maximal translocation of α_{2C} -AR to the cell surface in cell lines under these experimental conditions (Filipeanu et al., 2011a). This apparent long time required for the development of the full response may appear slow in comparison with the fast reactions observed in Raynaud's phenomenon, probably because the experiments were performed in transfected cell lines, which may mask the contribution of different multiple mechanisms involved in the temperature-sensitive α_{2C} -AR intracellular traffic. In these experimental conditions, the human α_{2C} -AR plasma membrane levels were enhanced by $73 \pm 9\%$ after exposure to 30°C, whereas the mouse and rat receptor cell surface levels were increased only by $33 \pm 6\%$ and $29 \pm 4\%$, respectively (Fig. 1A). In stark contrast, exposure to low temperature did not change the α_{2C} -AR plasma membrane levels in the OK cell line (Fig. 1A).

To test if the observed differences were caused by the expression in different cell lines or by intrinsic species differences in the receptor intracellular traffic, we determined the effects of low temperature on the transfected human and rat α_{2C} -AR in HEK293T cells and human coronary VSMCs, which both do not express the endogenous receptor. In these cell lines we found that at 37°C the plasma membrane levels of human receptor were much lower compared with the rat receptor levels, despite the comparable total amount of expressed receptors (Fig. 1, B and C). Exposure to low temperature had a much greater effect of enhancing human α_{2C} -AR plasma membrane levels in both cell lines ($73 \pm 9\%$ and $86 \pm 11\%$ in HEK293T and coronary VSMCs) compared with rat α_{2C} -AR ($36 \pm 6\%$ and $31 \pm 8\%$, respectively) (Fig. 1B). In fact, the effects of low temperature on the rat α_{2C} -AR plasma membrane levels were similar in rat tail VSMC artery, human coronary VSMCs, and HEK293T cells (compare Fig. 1, A and B). Also, confocal microscopy examination of subcellular localization demonstrated that rat α_{2C} -AR has a more prominent plasma membrane localization compared with human receptor at 37°C in human coronary VSMCs (Fig. 1D). Thus, our data suggest that the temperature-sensitive α_{2C} -AR transport to the cell surface is regulated in a specie-dependent manner.

In an attempt to determine the receptor characteristics underlying these traffic patterns we analyzed the amino acid sequence of α_{2C} -AR in several species. No major differences were observed in the receptor structure in the extracellular loops and transmembrane domains between human, rat, and mouse α_{2C} -AR. (Fig. 2A). Also, the amino acid compositions of the first and second intracellular loops were very similar in the human, rat, mouse, and opossum receptors (Fig. 2A). However, in the third intracellular loop and in the C-terminus, which are regions known to contain amino acid motifs essential for GPCR trafficking (Dong et al., 2007; Wu, 2012), several differences

Fig. 1. (A) The effects of temperature on the subcellular localization of α_{2C} -AR from different species. The receptor plasma membrane levels were determined in HEK293T cells transfected with human α_{2C} -AR, mouse, and rat VSMC from tail artery, and OK cells at 37°C (black columns) and at 30°C (white columns) using [3 H]RX821002 as previously described (Filipeanu et al., 2011a). $n = 8-12$ in each case from at least three different passages. * $P < 0.05$ and ** $P < 0.01$ at 30°C compared with 37°C. (B) The cell surface levels of human (left columns) and rat α_{2C} -AR (right columns) in HEK293T (top panel) and human coronary VSMC cells (bottom panel) at different temperatures (37°C and 30°C). $n = 6-12$ in each case; * indicates significant differences between 37°C and at 30°C. (C) The total α_{2C} -AR cellular levels at 37°C and 30°C in HEK293T cells (upper panel) and in human coronary VSMC (bottom panel) detected by Western blot using a receptor raised against receptor (Abcam). Similar results were obtained in two different experiments. NT, not-transfected cells. (D) Subcellular localization of human (left) and rat (right) α_{2C} -AR in human coronary VSMC at 37°C. Blue, nucleus staining using 4',6-diamidino-2-phenylindole. The images are representative from three independent electroporation experiments.

were clearly noticeable. First, many arginine-rich clusters are present in the third intracellular loop and C-terminus of α_{2C} -AR from higher mammals (Fig. 2A). In contrast, the opossum receptor is missing the cluster from the C-terminus (Fig. 2A) and its third intracellular loop has a higher percentage of alanine and histidine residues (Fig. 2A). The long arginine clusters from rodent and human α_{2C} -AR are organized as putative RXR motifs; when present in other proteins, such motifs act as an endoplasmic reticulum retention motif, preventing the transport of the proteins bearing these motifs to the cell surface. Indeed, these motifs are largely absent in the α_{2B} -AR third intracellular loop and C-terminus (Fig. 2B), which despite sharing over 80% homology with α_{2C} -AR, this does not change its plasma membrane levels after exposure to low temperature (Filipeanu et al., 2011a).

Fig. 2. (A) Alignment of α_{2C} -AR from different species. The amino acids shown in blue form the extracellular (EL) domains, the amino acids shown in brown form the transmembrane (TM) domains, and the amino acids shown in black constitute the intracellular (IC) loops. The amino acids differing from human sequence are underlined and italicized. The putative RXR motifs are shown in red bold characters. The four amino acids missing in the rat and mouse α_{2C} -AR third intracellular loop compared to human receptor are indicated in green. The alignment was performed using the ClustalW2 program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). (B) The effects of deletion of selected sequences on the human α_{2C} -AR temperature-dependent traffic. The receptor regions deleted are shown in green. In the α_{2C} -AR/ α_{2B} -AR chimeric construct, the domains shown in red from α_{2B} -AR were incorporated in the α_{2C} -AR structure between by using the BamHI restriction sites (residues shown in green in the bottom panel). The plasma membrane α_{2C} -AR levels were determined as in Fig. 1 and are expressed as the 30°C/37°C levels ratio. $n = 6$ –10 in each case; * $P < 0.05$ compared with the wild-type α_{2C} -AR human receptor.

We hypothesized that these RXR motifs may be responsible for the temperature-dependent transport of α_{2C} -AR to the plasma membrane. To test this hypothesis, we initially generated two human α_{2C} -AR deletion mutants. In the first mutant (α_{2C} -3iC), we deleted 125 amino acids from the third intracellular loop of human α_{2C} -AR, removing the putative eight RXR motifs located at this level. In the second mutant (α_{2C} -R9) we removed the last nine amino acids from the C-terminus, including the ⁴⁵⁴RRRRR⁴⁵⁸ sequence, which contains three putative RXR motifs (Fig. 2, A and B). Surprisingly, in transfected HEK293T cells, at 37°C these deletion mutants have similar cell surface levels as the wild-type human α_{2C} -AR (Fig. 2B). However, at 30°C, both mutants displayed statistically significant higher plasma membrane levels (Fig. 2B). Furthermore, a mutant carrying both deletions has higher plasma membrane levels compared with single deletion mutants at 30°C, but has the same levels as α_{2C} -AR at 37°C (Fig. 2B). These results suggest that at 37°C these RXR motifs are silent, but become functional at 30°C, preventing exaggerated receptor transport to the cell surface. Since we have previously shown that the α_{2B} -AR traffic is not temperature sensitive (Filipeanu et al., 2011a), we constructed a chimeric α_{2C} -AR that contained the third intracellular loop and the C-terminus of α_{2B} -AR (Fig. 2B). The corresponding chimera

further substantiated our conclusion, in that this construct had slightly elevated cell surface levels at 37°C, but exposure to low temperature (30°C) had no effect on its plasma membrane levels (Fig. 2B). These results taken together clearly implicate the RXR motifs in the third intracellular loop and the C-terminus of α_{3C} -AR as being responsible for the temperature-sensitive trafficking.

To precisely identify which arginine clusters modulate the α_{2C} -AR temperature-dependent trafficking, we performed progressive alanine mutagenesis. Surprisingly, individual disruption of any single RXR motif did not change the cell surface levels at 37°C or at 30°C (Table 1). These data suggest that these RXR motifs are redundant and multiple motifs are involved in the temperature-sensitive receptor trafficking. We further generated α_{2C} -AR mutants having multiple RXR motifs disrupted throughout the sequence and found that only mutants with disrupted arginine clusters in positions ²⁹⁴RRRRR²⁹⁸ and ⁴⁵⁴RRRRR⁴⁵⁸ have significantly enhanced plasma membrane receptor levels after exposure to low temperature, without significant changes at 37°C (Table 1). In fact, this pattern fully resembles the results obtained with α_{2C} -3ic and α_{2C} -R9 deletion individual and double mutants. Any additional mutation of other RXR motifs did not significantly change the cell surface levels of human α_{2C} -AR at 37°C or at 30°C in HEK293T cells.

TABLE 1

Identification of RXR motifs preventing exaggerated transport of α_{2C} -AR to the plasma membrane at 30°C

The underlined residues in human α_{2C} -AR were mutated from arginine to alanine using the specific primers indicated in the *Materials and Methods*. Subsequently, the mutants with single cluster disruption served as a template for generating double or triple mutants. The expected mutation was confirmed in each case by sequence analysis. The plasma membrane levels were determined by [3 H]RX821002 binding and the results are expressed as a percentage of α_{2C} -AR wild-type levels determined in the same experiment. $n = 8$ –12 in each case, from three independent transfections.

Receptors	37°C	30°C
	%	
With single cluster disrupted		
Wild-type	100	169 $\pm$ 6.2
240RTA242	96 $\pm$ 4.2	168 $\pm$ 8.4
294RRRAAR298	93 $\pm$ 7.3	175 $\pm$ 10.1
306RRA308	102 $\pm$ 6.7	166 $\pm$ 7.3
358RRRAAR364	107 $\pm$ 9.5	164 $\pm$ 8.5
454RRRAAR458	95 $\pm$ 5.5	176 $\pm$ 6.6
With two clusters disrupted		
240RTA242/293RRRAAR297	92 $\pm$ 6.8	173 $\pm$ 8.0
240RTA242/306RRA308	101 $\pm$ 3.1	168 $\pm$ 6.5
240RTA242/358RRRAAR364	97 $\pm$ 5.5	177 $\pm$ 7.5
240RTA242/454RRRAAR458	94 $\pm$ 6.8	173 $\pm$ 6.6
294RRRAAR298/306RRA308	104 $\pm$ 7.7	165 $\pm$ 8.2
294RRRAAR298/358RRRAAR364	101 $\pm$ 3.1	177 $\pm$ 6.2
294RRRAAR298/454RRRAAR458	103 $\pm$ 6.2	215 $\pm$ 7.4*
With three clusters disrupted		
294RRRAAR298/358RRRAAR364/454RRRAAR458	104 $\pm$ 7.8	221 $\pm$ 8.3*

* $P < 0.05$ compared with the wild-type human α_{2C} -AR receptor.

(Table 1). The plasma membrane α_{2C} -AR levels are directly correlated with the effects of receptor stimulation on cAMP levels (Filipeanu et al., 2011a; Jeyaraj et al., 2012). Accordingly, the effects of UK14304 (an α_2 -AR agonist) on forskolin-stimulated cAMP increases in α_{2C} -AR wild-type or $^{294}\text{RRRRR}^{298}/^{454}\text{RRRRR}^{458}$ - α_{2C} -AR-transfected HEK293T cells were minimal at 37°C (Table 2). In contrast, the effects of $^{294}\text{RRRRR}^{298}/^{454}\text{RRRRR}^{458}$ - α_{2C} -AR stimulation in cells exposed to low temperature were significantly greater (Table 2). The observed differences in the plasma membrane receptor levels and in the signaling abilities are not related to different total cellular expression levels of the receptor constructs as demonstrated by Western blot (Supplemental Fig. 1A). These data clearly demonstrate that the RXR motifs embedded in the arginine clusters from position $^{294}\text{RRRRR}^{298}$ and $^{454}\text{RRRRR}^{458}$ clusters of human α_{2C} -AR prevent the receptor transport at physiologic temperature, and this inhibitory effect is released at low temperature, resulting in enhanced traffic to the plasma membrane and augmented functional responses.

Two major mechanisms were proposed to underlie the endoplasmic reticulum retention by the RXR motifs, namely, retrograde transport through COPI vesicles mediated by interactions with the β -COP subunit and binding to 14-3-3 proteins (Okamoto and Shikano, 2011; Cunningham et al., 2012). However, 14-3-3 ζ overexpression did not change the human α_{2C} -AR plasma membrane levels at 37°C or at 30°C in transfected HEK293T cells (Fig. 3A). In contrast, downregulation of β -COP slightly reduced the α_{2C} -AR cell surface localization at 37°C, but greatly stimulated the effects of low temperature on the receptor trafficking (Fig. 3B). In agreement, we found that the $^{294}\text{RRRRR}^{298}/^{454}\text{RRRRR}^{458}$ - α_{2C} -AR mutant has diminished interactions with β -COP at 30°C compared with α_{2C} -AR wild type, as demonstrated by coimmunoprecipitation (Fig. 3C), indicating that the retrograde traffic through COPI vesicles plays a major role in preventing exaggerated α_{2C} -AR to plasma membrane at low temperature.

Still, rat α_{2C} -AR had larger plasma membrane levels at 37°C and displayed a reduced sensitivity after exposure to low temperature, although it contains the same arginine clusters in its structure. The $^{450}\text{RRRRR}^{454}$ cluster from the C-terminus is located in the same position in human and rat receptors, and thus is probably not responsible for the observed differences (Fig. 2A). In contrast, in rat α_{2C} -AR the $^{291}\text{RRRRR}^{296}$ cluster from the third intracellular loop is shifted three amino acids to the left due to the absence in the rat receptor of three residues, $^{268}\text{AG}^{269}$ and ^{291}A (Fig. 2A). To determine if strict spatial positioning of this arginine cluster is essential for temperature-sensitive intracellular trafficking, we generated mutants of the rat α_{2C} -AR, in which the missing amino acids were inserted at the same positions as in the human receptor. Both $^{268}\text{AG}^{269}$ - and ^{291}A -rat α_{2C} -AR mutants had significant lower plasma membrane levels at 37°C in HEK293T cells (Fig. 4A) despite having similar total receptor levels as detected by Western blot (Supplemental Fig. 1B). The cell surface levels of these mutants were increased by exposure to low temperature with significantly greater extent compared with the wild-type rat receptor (Fig. 4A). Furthermore, the mutant carrying the insertion of all

TABLE 2

The effects of human α_{2C} -AR wild-type and $^{294}\text{RRRRR}^{298}/^{454}\text{RRRRR}^{458}$ - α_{2C} -AR mutant stimulation on cAMP levels

HEK293T cells transiently transfected with 3 $\mu\text{g}/\text{plate}$ of each receptor construct for 48 hours and subsequently starved for 24 hours. The cells were then treated with 10^{-8} M UK14304 for 5 minutes, followed by incubation with forskolin (3 μM) for 15 minutes. The cAMP levels were determined as previously described (Filipeanu et al., 2011a,b) using the cAMP enzyme immunoassay system. $n = 6$ in each case from three independent transfections.

α_{2C} -AR Wild-Type Receptor		$^{294}\text{RRRRR}^{298}/^{454}\text{RRRRR}^{458}$ - α_{2C} -AR	
37°C	30°C	37°C	30°C
%			
84.89 $\pm$ 13.5	57.26 $\pm$ 8.6	81.24 $\pm$ 12.3	24.62 $\pm$ 10.1*

* $P < 0.05$ compared with the wild-type human α_{2C} -AR receptor.

Fig. 3. (A) Top: the cellular levels of 14-3-3ζ in pcDNA3.1 and 14-3-3ζ transfected cells. Bottom: the effects of 14-3-3ζ overexpression on the human α_{2C}-AR traffic in HEK293T cells, as determined by specific [³H]RX821002 binding. *n* = 6 from three independent transfections. (B) The cellular levels of β-COP in cells transfected with scrambled small hairpin RNA (shRNA) and β-COP shRNA. Bottom: plasma membrane levels of α_{2C}-AR in control cells and in cells with reduced β-COP levels. The experiments were performed in a similar manner as in (A). *n* = 6 in each case from three independent transfections; **P* < 0.05 compared with scrambled shRNA. (C) Interactions between human wild-type α_{2C}-AR or 294RRRAAR^{298/458} α_{2C}-AR mutant with β-COP at 37°C and 30°C in HEK293T cells detected by coimmunoprecipitation. HEK293T cells were transfected with the indicated receptors and 3 days later the cells were solubilized and immunoprecipitated with GFP antibody as previously described (Filipeanu et al., 2011a). The immunoprecipitates (20 μg/lane) were separated by 10% SDS-PAGE and the β-COP levels were revealed by Western blotting. IB, immunoblotted; IP, immunoprecipitated; NT, not-transfected cells. The experiment shown is representative from three independent transfections. On the left, the quantification of these three independent experiments is shown, *n* = 3; **P* < 0.05.

of these three amino acids located proximal to 291RRRRR²⁹⁶ had similar plasma membrane levels at 37°C and displayed the same temperature sensitivity as the human receptor (Fig. 4A). Interestingly, the fourth amino acid missing in the rat α_{2C}-AR third intracellular loop compared with the human receptor is located distal to the 291RRRRR²⁹⁶ cluster in position 325G (Fig. 2A). Supporting the idea that differences in the temperature sensitivity of human and rat α_{2C}-AR are due to strict spatial requirements of this arginine cluster, the cell surface levels of the rat 325G α_{2C}-AR mutant are not different compared with the rat wild-type receptor (Fig. 4A). In agreement with the radioligand binding determinations, in transfected HEK293T cells at 37°C, stimulation of the rat α_{2C}-AR reduced the cAMP levels to a greater extent compared with human receptors (Fig. 4B). In contrast, stimulation of 268AG^{269/291}A rat α_{2C}-AR had minimal effects on cAMP levels, as in the case of human α_{2C}-AR (Fig. 4B). Thus, the insertion of these three amino acids into the rat

α_{2C}-AR appears to have restored temperature-sensitive trafficking to the levels seen in the human α_{2C}-AR.

Regulation of GPCR intracellular trafficking involves interactions with accessory proteins along the biosynthetic pathway. To determine the regulatory proteins responsible for the trafficking differences observed between human and rat α_{2C}-AR, we performed a proteomic analysis in rat and human α_{2C}-AR-transfected HEK293T cells. From this analysis three proteins were identified to have differences in the interactions with the two receptors, namely, pontin (also known as RuvB-like 1 or Tip49), p97 (also named VCP), and sorting nexin 9 (Fig. 5A; Table 3). The first two, pontin and p97, belong to the group of AAA+ ATPases, a class of proteins associated with a variety of cellular processes ranging from DNA replication and transcription regulation to protein degradation. The interactions of human α_{2C}-AR with pontin were confirmed by coimmunoprecipitation (Fig. 5B), and we confirmed that these interactions were decreased at low temperature (Fig. 5B). Rat α_{2C}-AR displayed lower interactions with pontin, which were

Fig. 4. (A) The four amino acids missing in the rat α_{2C}-AR third intracellular loop were inserted by site mutagenesis and the cell surface levels of these mutants were determined at 37°C and 30°C by [³H]RX821002 binding. 269GA²⁷⁰ and 291A mutants displayed significantly lower plasma membrane levels at 37°C, and greater enhancement after exposure to low temperature. The triple 269GA^{270/291}A mutant behaved similarly to human α_{2C}-AR. The insertion of 326G amino acid has no effect on the receptor plasma membrane levels. *n* = 6–8 in each case; **P* < 0.05 compared with wild-type α_{2C}-AR rat receptor. (B) The effects of human, rat, and 269GA^{270/291}A rat α_{2C}-AR at 37°C on forskolin-induced cAMP increases at 37°C. The experiments were performed as previously described, *n* = 6 in each case from two different transfections; **P* < 0.05.

not temperature sensitive (Fig. 5B). Also, supporting the role of pontin in the regulation of α_2C -AR intracellular traffic, we found that the ²⁶⁸AG²⁶⁹/291A rat α_2C -AR mutant interacts with this protein in a temperature-sensitive manner similar to the human receptor (Fig. 5B), indicating once again that the three residues are responsible for the differences in the temperature sensitivity between human and rat receptors. Furthermore, HEK293T cells with genetically reduced pontin levels and HEK293T cells treated with the recently described pontin inhibitor rottlerin (5 μ M) (Elkaim et al., 2012), show significantly enhanced human α_2C -AR plasma membrane levels at 37°C. However, in HEK293T cells with genetically reduced levels of pontin, the cell surface levels of rat α_2C -AR were not changed (Fig. 6A), and neither did the pharmacological inhibition of pontin activity using rottlerin change the plasma membrane levels of rat α_2C -AR (Fig. 6A). The effects of reduced pontin activity were not observed at 30°C (Fig. 6A). Genetic or pharmacological inhibition of pontin activity also specifically enhanced human α_2C -AR signaling by potentiating the effects of receptor stimulation on p-ERK 1/2 (Fig. 6B) and on inhibition of cAMP levels (Fig. 6C) at 37°C.

Fig. 5. (A) The histograms representing the volumes of specific spot interactions with human and rat α_2C -AR in HEK293T cells determined with the DeCyder software. Subsequently, the spot was picked, subjected to trypsin digestion, and the peptides were subjected to tandem MS analysis. The liquid chromatography–MS data were analyzed using the Proteome Discoverer (Thermo Fisher Scientific) 1.4 using the Human SwissProt database. On the right, the peptides obtained during analysis are shown in red, identifying pontin (also named RuvBL-1 or TIP49) as an interacting partner of human α_2C -AR. The experiments were repeated three times and the statistics are shown in the bar graph. * $P < 0.05$. (B) Validation of proteomics data by coimmunoprecipitation. GFP-tagged human, rat, and ²⁶⁸GA²⁷⁰/²⁹¹A rat mutant α_2C -AR were transfected in HEK293T cells. Two days later the cells were serum starved and exposed to 37°C or 30°C for 18 hours, and then were processed as described in Fig. 3. Pontin levels in immunoprecipitates and in inputs were determined by Western blot. IB, immunoblotted; IP, immunoprecipitated; NT, not-transfected cells. Similar results were obtained in two other experiments.

TABLE 3

Proteins identified to interact differentially with human and rat α_2C -AR in HEK293T cells

Human and rat GFP-tagged α_2C -AR were transiently transfected in HEK293T cells (8 μ g/plate) and the cells were starved 24 hours before the experiment. Three days after transfection the cells were lysed and incubated with Anti-GFP mAb-Agarose overnight. Dimensional gel electrophoresis labeling and analysis were performed as previously described (Filipeanu et al., 2011b). Fluorophore-labeled protein gels were scanned, detected, and analyzed as described in the *Materials and Methods*. Implicated spots were subjected to liquid chromatography–MS and analysis as described in the *Materials and Methods* and spot verification was performed by Western blot.

Protein	Molecular Mass	Score	Peptides Matched
Pontin (RUVBL1, TIP49)	50,524	762	40
P97 (PCP)	89,950	2180	145
SNX9 (SH3PX1)	66,903	100	4

Discussion

GPCR intracellular traffic plays fundamental roles in targeting the receptors to the functional site and modulates the amplitude of cellular signaling to receptor stimulation. The temperature-sensitive α_2C -AR export to the plasma membrane in VSMCs is responsible for the exaggerated vasoconstriction in response to cold and emotional stress observed in Raynaud's phenomenon. The present results unveil several novel features of this receptor's distinct intracellular trafficking. Most importantly, we found that the effects of low temperature on α_2C -AR are species dependent, being absent in lower mammals such as the opossum. This differential temperature sensitivity is mediated by specific arginine clusters organized as RXX motifs in the third intracellular loop and the C-terminus of the receptor. Surprisingly, we found that disruption of these RXX motifs does not change the plasma membrane receptor levels at 37°C, suggesting that multiple mechanisms may contribute to α_2C -AR intracellular retention at physiologic temperature. However, the roles of specific RXX motifs became evident at reduced temperature, as the mutation of motifs in positions ²⁹⁴RRRRR²⁹⁸ and ⁴⁵⁸RRRRR⁴⁶² prevent the exaggerated transport of human α_2C -AR to the cell surface. These two clusters operate in a redundant manner and disruption of both is required for maximal transport to the plasma membrane in response to cold.

In contrast to the well characterized mechanisms involved in GPCR internalization, the motifs involved in GPCR traffic from the endoplasmic reticulum to the plasma membrane remain poorly characterized (Wu, 2012). A highly conserved F(x)6LL motif located in the proximal C-terminus of many GPCRs is a common motif mediating the transport from the endoplasmic reticulum to the plasma membrane (Duvernay et al., 2004). Another motif present in most GPCRs is a cluster of 3–5 basic residues (arginine or lysine) in the third intracellular loop, which interacts with the Sec24 subunit of the cargo complex to stimulate receptor export (Dong et al., 2012). Both these motifs are embedded in the α_2C -AR structure at positions ⁴⁴³FRRSFKHIL⁴⁵² and ³⁶⁸RRK³⁷¹. However, these motifs are involved in the general GPCR transport to the plasma membrane, as indicated by the common presence in many GPCRs, including α_2B -AR. This is the closest homolog to α_2C -AR, but it does not display temperature-sensitive trafficking. In addition to export motifs from intracellular domains, the YS motif located in the N-terminus was demonstrated to mediate the traffic of all three α_2 -AR from the Golgi to the plasma membrane (Dong and Wu, 2006). Regarding α_2C -AR, a hydrophobic

Fig. 6. (A) The effects of downregulation of pontin on the human and rat α_{2C} -AR plasma membrane levels. A clone of HEK293T cells with reduced pontin levels ($23 \pm 12\%$ from control, $n = 4$) was generated using a small hairpin RNA (shRNA) lentiviral construct and puromycin selection. The receptors were transfected for 72 hours in control or pontin-depleted cells. When present, Rottlerin (pontin inhibitor, $5 \mu\text{M}$) was incubated for 18 hours. $n = 9$ from three independent cell passages; $*P < 0.05$ compared with control cells. (B) The effects of pontin depletion on α_{2C} -AR stimulation of p-ERK 1/2 phosphorylation at 37°C . The cells were processed as in (A), and 72 hours post-transfection were stimulated for 5 minutes with indicated concentrations of specific α_2 -AR agonist UK14304. Black circles indicate the effects obtained in control cells and open circles indicate the effects obtained in HEK293T cells with reduced pontin levels. $n = 3$ from three independent experiments; $*P < 0.05$ compared with control cells. (C) The effects of reducing pontin cellular levels (LV pontin) on α_{2C} -AR-mediated decrease in cAMP levels at 37°C . The cells were pretreated with UK14304 (10^{-8} M) for 5 minutes and then stimulated with forskolin (FSK; $3 \mu\text{M}$) for 15 minutes, and cAMP was determined as previously described (Filipeanu et al., 2011 a,b). $n = 9$ from three independent experiments; $*P < 0.05$ compared with the control cells.

motif, ALAAALAAAA, from the N-terminus of the receptor has been shown to contribute to its endoplasmic reticulum retention (Angelotti et al., 2010). However, this motif appears not to mediate the temperature-sensitive receptor traffic because it is present also in mouse and rat α_{2C} -AR. In contrast to other export motifs, our results demonstrate that $^{294}\text{RRRRR}^{298}$ and $^{458}\text{RRRRR}^{462}$ are unique human α_{2C} -AR features, preventing the exaggerated receptor transport to the cell surface at low temperature.

Two major mechanisms were demonstrated to modulate in opposite ways the ability of RXR motifs to mediate the endoplasmic reticulum retention. The β -COP subunit of the COPI complex recognizes RXR motifs stimulating the retrograde traffic from Golgi to endoplasmic reticulum, whereas 14-3-3 proteins assist motif masking and promote export trafficking

(Dong et al., 2007). Both these mechanisms were shown to modulate subcellular localization of different members of the GPCR family, such as PAR2 and GPR15 (Okamoto and Shikano, 2011; Cunningham et al., 2012). However, our results indicate that 14-3-3 ζ is not involved in temperature-sensitive α_{2C} -AR transport. This finding can be explained by the absence of the distal phosphorylation sites adjacent to the RXR motifs required for interactions with 14-3-3 proteins in α_{2C} -AR. In contrast, reducing β -COP cellular levels enhanced the effects of low temperature on the α_{2C} -AR transport, whereas it had a minimal effect at 37°C . Disruption of the $^{294}\text{RRRRR}^{298}$ and $^{458}\text{RRRRR}^{462}$ regulating the temperature-sensitive α_{2C} -AR traffic attenuated the interactions between receptor and β -COP only at 30°C , explaining the enhanced plasma membrane localization of this mutant at reduced temperature.

However, unlike the opossum receptor, rat and mouse α_{2C} -AR have equivalent arginine clusters, although the temperature sensitivity of these receptors is reduced. This pattern might be due to enhanced plasma membrane localization of rat and mouse receptors at 37°C . Because the C-terminus arginine cluster $^{458}\text{RRRRR}^{462}$ is similar in all three species, we focused on the $^{294}\text{RRRRR}^{298}$ cluster from the third intracellular loop. Our insertion experiments demonstrate that indeed the absence in the rat receptor of the three amino acids located at $^{269}\text{GA}^{270}/^{291}\text{A}$ proximal to this cluster are responsible for enhanced rat α_{2C} -AR plasma membrane localization at 37°C . Notably, insertion of these residues in rat α_{2C} -AR did not change the cell surface receptor levels at low temperature, resembling the traffic pattern of the human receptor. In contrast, insertion of the fourth missing amino acid in the rat α_{2C} -AR third intracellular loop, located distal to this cluster, ^{356}G , has no effect on receptor traffic, suggesting that strict spatial positioning of the $^{294}\text{RRRRR}^{298}$ cluster is required to limit human α_{2C} -AR transport to the cell surface. These results indicate that a rodent model is not suitable in studying the pathology of Raynaud's phenomenon. Furthermore, only a few studies have analyzed the fine differences between GPCRs from different species, focusing mostly on the different receptor affinities generated by evolutionary mutations in the transmembrane domains (Schattauer et al., 2012; Scott et al., 2013; Strasser et al., 2013). To the best of our knowledge, this is the first study demonstrating major species-dependent intracellular traffic differences in the GPCR family, and this finding should be considered in future studies on receptor traffic, subcellular localization, and signaling.

A major mechanism involved in GPCR localization is represented by receptor interactions with specialized intracellular accessory proteins generically named molecular chaperones (Dong et al., 2007). In the last few years, specific molecular chaperones for different GPCRs have been identified, and we and others have demonstrated that the cellular levels of these accessory proteins can affect the subcellular localization and signaling of the chaperoned receptor (Williams and Devi 2010; Filipeanu et al., 2011b). Based on the observed differences between human and rat α_{2C} -AR plasma membrane levels at 37°C , we performed a proteomic analysis to identify the different protein interactions of these receptors. We found that pontin, p97, and nexin as accessory proteins interact differentially with the two receptors. Nexin has been previously shown to regulate the intracellular traffic of P2Y1 and PAR1 in a retromer-independent manner (Gullapalli et al., 2006;

Nisar et al., 2010). In contrast, the involvement of pontin and p97 in the modulation of GPCR traffic has not been previously reported. Both pontin and p97 are members of AAA+ ATPases, a large and diverse superfamily characterized by the presence of a highly conserved ATPase module of 200–250 amino acids (Snider et al., 2008; Grigoletto et al., 2011). The exact roles of each member of this family are diverse and not fully characterized, but include chromatin remodeling, transcription regulation, protein unfolding, and degradation, and many of its members, including p97 and pontin, are overexpressed in cancer or neurodegenerative disorders (Jha and Dutta, 2009; Nano and Houry, 2013). Out of these three proteins we selected pontin to further study its role in modulation of α_{2C} -AR temperature-sensitive traffic because recently autoantibodies against pontin were detected in systemic sclerosis, a disease that may be associated with Raynaud's phenomenon (Kaji et al., 2014). We found that the direct interactions with human α_{2C} -AR in a temperature-sensitive manner were confirmed by coimmunoprecipitation, and pontin did not change the total cellular receptor levels. These results indicate that pontin effects on human α_{2C} -AR are not related to changes in transcription or degradation. However, reducing pontin activity by genetic or pharmacological approaches strongly enhanced human receptor plasma membrane levels and signaling at physiologic temperature, indicating that these interactions limit the α_{2C} -AR transport to the cell surface at 37°C. Our data are in agreement with recent findings in patients with systemic sclerosis and suggest that decreased pontin activity may represent a diagnostic biomarker for Raynaud's phenomenon (Kaji et al., 2014). Interestingly, pontin, together with its partner reptin, has been shown to interact with HSP90 (Izumi et al., 2012). We previously demonstrated that inhibition of HSP90 enhanced the α_{2C} -AR plasma membrane levels at 37°C (Filipeanu et al., 2011a). Together with the present demonstration that pontin limits receptor transport at physiologic temperature, we propose that multiple interactions with various chaperones along the biosynthetic pathway are responsible for the unique temperature-sensitive pattern of human α_{2C} -AR. Recently, cAMP-mediated Epac activation was proposed to mediate α_{2C} -AR plasma membrane delivery through a Rap1-Rho-actin-dependent mechanism (Jeyaraj et al., 2012; Michel and Insel, 2012). However, these effects were also observed at 37°C and with both human and murine receptors, and thus seem to mediate receptor traffic from the Golgi apparatus to the cell surface. In contrast, HSP90 and pontin appear to be specific mediators of temperature-sensitive α_{2C} -AR intracellular traffic and may constitute valuable biomarkers to identify people predisposed to Raynaud's phenomenon. Overall, our data represent the first demonstration of unique mechanisms underlying the temperature sensitivity of human α_{2C} -AR trafficking, particularly the prevention of exaggerated traffic to the plasma membrane at reduced temperature by specific arginine clusters from the third intracellular loop and C terminus through retrograde traffic mediated by COPI vesicles and receptor interactions in a temperature-sensitive manner with pontin.

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Authorship Contributions

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References

- Angelotti T, Daunt D, Shcherbakova OG, Kobilka B, and Hurt CM (2010) Regulation of G-protein coupled receptor traffic by an evolutionary conserved hydrophobic signal. *Traffic* **11**:560–578.
- Bailey SR, Eid AH, Mitra S, Flavahan S, and Flavahan NA (2004) Rho kinase mediates cold-induced constriction of cutaneous arteries: role of α_{2C} -adrenoceptor translocation. *Circ Res* **94**:1367–1374.
- Brand FN, Larson MG, Kannel WB, and McGuirik JM (1997) The occurrence of Raynaud's phenomenon in a general population: the Framingham Study. *Vasc Med* **2**:296–301.
- Bylund DB, Blaxall HS, Iversen LJ, Caron MG, Lefkowitz RJ, and Lomasney JW (1992) Pharmacological characteristics of α_2 -adrenergic receptors: comparison of pharmacologically defined subtypes with subtypes identified by molecular cloning. *Mol Pharmacol* **42**:1–5.
- Chotani MA, Flavahan S, Mitra S, Daunt D, and Flavahan NA (2000) Silent α_{2C} -adrenergic receptors enable cold-induced vasoconstriction in cutaneous arteries. *Am J Physiol Heart Circ Physiol* **278**:H1075–H1083.
- Cunningham MR, McIntosh KA, Pediani JD, Robben J, Cooke AE, Nilsson M, Gould GW, Mundell S, Milligan G, and Plevin R (2012) Novel role for proteinase-activated receptor 2 (PAR2) in membrane trafficking of proteinase-activated receptor 4 (PAR4). *J Biol Chem* **287**:16656–16669.
- Daunt DA, Hurt C, Hein L, Kallio J, Feng F, and Kobilka BK (1997) Subtype-specific intracellular trafficking of α_2 -adrenergic receptors. *Mol Pharmacol* **51**:711–720.
- Deshotel MR, Xia H, Sriramula S, Lazartigues E, and Filipeanu CM (2014) Angiotensin II mediates angiotensin converting enzyme type 2 internalization and degradation through an angiotensin II type 1 receptor-dependent mechanism. *Hypertension* **64**:1368–1375.
- Dong C, Filipeanu CM, Duvernay MT, and Wu G (2007) Regulation of G protein-coupled receptor export trafficking. *Biochim Biophys Acta* **1768**:853–870.
- Dong C, Nichols CD, Guo J, Huang W, Lambert NA, and Wu G (2012) A triple arg motif mediates α_{2B} -adrenergic receptor interaction with Sec24C/D and export. *Traffic* **13**:857–868.
- Dong C and Wu G (2006) Regulation of anterograde transport of α_2 -adrenergic receptors by the N termini at multiple intracellular compartments. *J Biol Chem* **281**:38543–38554.
- Duvernay MT, Filipeanu CM, and Wu G (2005) The regulatory mechanisms of export trafficking of G protein-coupled receptors. *Cell Signal* **17**:1457–1465.
- Duvernay MT, Zhou F, and Wu G (2004) A conserved motif for the transport of G protein-coupled receptors from the endoplasmic reticulum to the cell surface. *J Biol Chem* **279**:30741–30750.
- Elkaim J, Castroviejo M, Bennani D, Taouji S, Allain N, Laguerre M, Rosenbaum J, Dessolin J, and Lestienne P (2012) First identification of small-molecule inhibitors of Pontin by combining virtual screening and enzymatic assay. *Biochem J* **443**:549–559.
- Filipeanu CM, de Vries R, Danser AH, and Kapusta DR (2011a) Modulation of α_{2C} -adrenergic receptor temperature-sensitive trafficking by HSP90. *Biochim Biophys Acta* **1813**:346–357.
- Filipeanu CM, Guidry JJ, Leonard ST, and Winsauer PJ (2011b) $\Delta 9$ -THC increases endogenous AHA1 expression in rat cerebellum and may modulate CB1 receptor function during chronic use. *J Neurochem* **118**:1101–1112.
- Filipeanu CM, Zhou F, Fugetta EK, and Wu G (2006) Differential regulation of the cell-surface targeting and function of β - and α_1 -adrenergic receptors by Rab1 GTPase in cardiac myocytes. *Mol Pharmacol* **69**:1571–1578.
- Flavahan NA (2008) Regulation of vascular reactivity in scleroderma: new insights into Raynaud's phenomenon. *Rheum Dis Clin North Am* **34**:81–87.
- Freedman RR, Baer RP, and Mayes MD (1995) Blockade of vasospastic attacks by α_2 -adrenergic but not α_1 -adrenergic antagonists in idiopathic Raynaud's disease. *Circulation* **92**:1448–1451.
- Grigoletto A, Lestienne P, and Rosenbaum J (2011) The multifaceted proteins Reptin and Pontin as major players in cancer. *Biochim Biophys Acta* **1815**:147–157.
- Gullapalli A, Wolfe BL, Griffin CT, Magnuson T, and Trejo J (2006) An essential role for SNX1 in lysosomal sorting of protease-activated receptor-1: evidence for retromer-, Hrs-, and Tsg101-independent functions of sorting nexins. *Mol Biol Cell* **17**:1228–1238.
- Hurt CM, Feng FY, and Kobilka B (2000) Cell-type specific targeting of the α_{2C} -adrenoceptor. Evidence for the organization of receptor microdomains during neuronal differentiation of PC12 cells. *J Biol Chem* **275**:35424–35431.
- Izumi N, Yamashita A, Hirano H, and Ohno S (2012) Heat shock protein 90 regulates phosphatidylinositol 3-kinase-related protein kinase family proteins together with the RUVBL1/2 and Tel2-containing co-factor complex. *Cancer Sci* **103**:50–57.
- Jeyaraj SC, Unger NT, Eid AH, Mitra S, Paul El-Dahdah N, Quilliam LA, Flavahan NA, and Chotani MA (2012) Cyclic AMP-Rap1A signaling activates RhoA to induce α_{2C} -adrenoceptor translocation to the cell surface of microvascular smooth muscle cells. *Am J Physiol Cell Physiol* **303**:C499–C511.
- Jha S and Dutta A (2009) RVB1/RVB2: running rings around molecular biology. *Mol Cell* **34**:521–533.
- Johnson JM and Kellogg DL, Jr (2010) Thermoregulatory and thermal control in the human cutaneous circulation. *Front Biosci (Schol Ed)* **2**:825–853.
- Kaji K, Fertig N, Medsger TA, Jr, Satoh T, Hoshino K, Hamaguchi Y, Hasegawa M, Lucas M, Schnure A, and Ogawa F et al. (2014) Autoantibodies to RuvBL1 and

- RuvBL2: a novel systemic sclerosis-related antibody associated with diffuse cutaneous and skeletal muscle involvement. *Arthritis Care Res (Hoboken)* **66**:575–584.
- Michel MC and Insel PA (2012) Can you blame cold feet on Epac (and Rap1A)? Focus on “cyclic AMP-Rap1A signaling activates RhoA to induce α_{2C} -adrenoceptor translocation to the cell surface of microvascular smooth muscle cells”. *Am J Physiol Cell Physiol* **303**:C488–C489.
- Morrison SF (2011) 2010 Carl Ludwig Distinguished Lectureship of the APS Neural Control and Autonomic Regulation Section: Central neural pathways for thermoregulatory cold defense. *J Appl Physiol* **110**:1137–1149.
- Nano N and Houry WA (2013) Chaperone-like activity of the AAA+ proteins Rvb1 and Rvb2 in the assembly of various complexes. *Philos Trans R Soc Lond B Biol Sci* **368**:20110399.
- Nguyen VA, Eisendle K, Gruber I, Hugl B, Reider D, and Reider N (2010) Effect of the dual endothelin receptor antagonist bosentan on Raynaud's phenomenon secondary to systemic sclerosis: a double-blind prospective, randomized, placebo-controlled pilot study. *Rheumatology (Oxford)* **49**:583–587.
- Nisar S, Kelly E, Cullen PJ, and Mundell SJ (2010) Regulation of P2Y1 receptor traffic by sorting Nexin 1 is retromer independent. *Traffic* **11**:508–519.
- Okamoto Y and Shikano S (2011) Phosphorylation-dependent C-terminal binding of 14-3-3 proteins promotes cell surface expression of HIV co-receptor GPR15. *J Biol Chem* **286**:7171–7181.
- Petaja-Repo UE, Hogue M, Laperriere A, Walker P, and Bouvier M (2000) Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. *J Biol Chem* **275**:13727–13736.
- Pope J, Fenlon D, Thompson A, Shea B, Furst D, Wells G, and Silman A (2000) Ketanserin for Raynaud's phenomenon in progressive systemic sclerosis. *Cochrane Database Syst Rev* **2**:CD000954.
- Pope JE (2007) The diagnosis and treatment of Raynaud's phenomenon: a practical approach. *Drugs* **67**:517–525.
- Prete M, Fatone MC, Favoino E, and Perosa F (2014) Raynaud's phenomenon: from molecular pathogenesis to therapy. *Autoimmun Rev* **13**:655–667.
- Raynaud M (1862) *Local Asphyxia and Symmetrical Gangrene of the Extremities*, L. Leclerc, Paris.
- Re M, Pampillo M, Savard M, Dubuc C, McArdle CA, Millar RP, Conn PM, Gobeil F, Jr, Bhattacharya M, and Babwah AV (2010) The human gonadotropin releasing hormone type I receptor is a functional intracellular GPCR expressed on the nuclear membrane. *PLoS ONE* **5**:e11489.
- Romanovsky A (2014) Skin temperature: its role in thermoregulation. *Acta Physiol (Oxf)* **210**:498–507.
- Schattauer SS, Miyatake M, Shankar H, Zietz C, Levin JR, Liu-Chen LY, Gurevich VV, Rieder MJ, and Chavkin C (2012) Ligand directed signaling differences between rodent and human κ -opioid receptors. *J Biol Chem* **287**:41595–41607.
- Scott JS, Brocklehurst KJ, Brown HS, Clarke DS, Coe H, Groombridge SD, Laber D, MacFaul PA, McKerrecher D, and Schofield P (2013) Conformational restriction in a series of GPR119 agonists: differences in pharmacology between mouse and human. *Bioorg Med Chem Lett* **23**:3175–3179.
- Snider J, Thibault G, and Houry WA (2008) The AAA+ superfamily of functionally diverse proteins. *Genome Biol* **9**:216.
- Strasser A, Wittmann HJ, Buschauer A, Schneider EH, and Seifert R (2013) Species-dependent activities of G-protein-coupled receptor ligands: lessons from histamine receptor orthologs. *Trends Pharmacol Sci* **34**:13–32.
- Williams D and Devi LA (2010) Escorts take the lead molecular chaperones as therapeutic targets. *Prog Mol Biol Transl Sci* **91**:121–149.
- Wood HM and Ernst ME (2006) Renin-angiotensin system mediators and Raynaud's phenomenon. *Ann Pharmacother* **40**:1998–2002.
- Wu G (2012) Regulation of post-Golgi traffic of G protein-coupled receptors. *Subcell Biochem* **63**:83–95.

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